INTERNATIONAL WORKSHOP
ON
STRUCTURAL AND FUNCTIONAL
ASPECTS OF THE
CHOLINERGIC SYNAPSE

Neve-Ilan Guest House, near Jerusalem, ISRAEL

August 30 - Sept. 4, 1987

Scientific Committee

G. Amitai (*Ness-Ziona*)
E. Kosower (*Tel-Aviv*)
D. Michaelson (*Tel-Aviv*)
R. Rahamimoff (*Jerusalem*)
I. Silman (*Rehovoth*)
H. Soreq (*Jerusalem*)

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The conference dealt with recent developments in the structure and function of the nicotinic acetylcholine receptor and acetylcholinesterase. During the four day period, over 50 talks and 31 poster sessions were presented. The high caliber of the presentations combined with the limited number of attendees, and secluded site, allowed for much fruitful interaction and in-depth discussions. The sessions were divided into five major categories: "Synaptogenesis and Regulation," "Acetylcholinesterase," "Presynaptic Mechanisms," "Signal Transduction and Modulation," and "Acetylcholine Receptor."

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- The Israel Institute for Psychobiology (Charles E. Smith Family Foundation).

- Maurice & Gabriella Goldschleger Conference Foundation at the Weizmann Institute of Science.

- Committee for Cell Biology and Biophysics of IUPAB.

- Israel Academy of Sciences and Humanities.

- Center for Neurosciences and Behavior of the Weizmann Institute of Science.

- International Union of Biochemistry.

- International Brain Research Organization.

- Conference Committee of the Hebrew University of Jerusalem.

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GENERAL INFORMATION

Lectures:

Projection equipment will be provided for 2"x 2" slides, for 16mm film and for overhead projector transparencies (25 x 25 cm). All speakers are requested to give their slides to the projectionist before their session.

Posters:

Poster authors are requested to put up their posters on Monday Aug 31, 7.30-8.30 a.m., and be present by their posters on Monday evening between 20.00 and 21.00. Posters will be displayed throughout the whole meeting.

Meal Times:

Breakfast	...............from	07.30
Lunch	Monday, Tuesday	13.00
	Wednesday, Thursday	12.30
Dinner	...............from	18.45

Name Badges:

Participants are requested to wear their name badges at all times whilst at Neve Ilan.

Secretariat and travel desk:

Will be open daily from 8.30. Please contact "Kenes" representative.

Social activities:

Several special events are planned:
Sunday   20.30 - Mixer Buffet
Monday   21.15 - Lecture on the Dead Sea Scrolls
Wednesday   13.30 - Excursion to Jerusalem
Thursday   18.00 - Closing dinner at the
                      National Palace Hotel Roof Top Gardens Restaurant
                      Jerusalem.
                      Participants are advised to bring
                      a sweater or jacket as it can be
                      cool in the evening in Jerusalem.

Participation in all the social events is covered by the registration fee.

Check out Time:

All participants are kindly requested to check out on Friday Sept, 4 by 9.00 a.m.

PROGRAM

Sunday, August 30th

18:00   Registration

20:30   Mixer

Monday, August 31st

SYNAPTOGENESIS & REGULATION (I)

CHAIRMAN: S. FUCHS (*Rehovot*)

08:30   J.-P. CHANGEUX (*Paris*)
A molecular approach to the development of the rat neuromuscular junction

09:00   S. ROTSHENKER (*Jerusalem*)
Multiple modes and sites for the regulation of motor axonal growth

09:30   R. WERMAN (*Jerusalem*)
Intracellular accumulation of acetylcholine can lead to neuronal death

10:00   M. BRZIN (*Ljubljana*)
The appearance of two distinct types of subsynaptic specialization bearing AChE activity in regenerating rat muscle

10:30   Coffee

11:00   U.J. McMAHAN (*Stanford*)
Agrin: identification and characterization

11:20   B.G. WALLACE (*Stanford*)
Agrin: mechanism of action

11:40   L. ANGLISTER (*Jerusalem*)
Acetylcholinesterase in regenerating neuromuscular junctions

12:00   Z. VOGEL (*Rehovot*)
Role of neurotrophic factor(s) and calcium channels in the regulation of acetylcholinesterase in cultured muscle

12:30   End of Session

13:00   Lunch

SYNAPTOGENESIS AND REGULATION (II)

CHAIRMAN: U.J. McMAHAN (*Stanford*)

13:00   M-m. POO (*New Haven*)
Early minutes of synaptogenesis: a physicochemical view

16:30   A. SHAINBERG (*Ramat Gan*)
Changes in the levels of acetylcholine receptors are mediated by the calcium concentration in the sarcoplasmic reticulum

17:00   Coffee

17:30	H. MEIRI (*Jerusalem*) *Short and long term effects of aluminum on synaptic transmission and on the activity of cultured nerve cells*
18:00	D. MICHAELSON (*Tel-Aviv*) *Antibodies to cholinergic neurons in Alzheimer's Disease*
18:20	End of session
18:45	Dinner
20:00	Poster session
21.15	Lecture on the Dead Sea Scrolls, M. Broshi.

Tuesday, September 1st

ACETYLCHOLINESTERASE

CHAIRMAN: M. BRZIN (*Ljubljana*)

08:30	J. MASSOULIE (*Paris*) *Molecular diversity of cholinesterases*
09:00	P. TAYLOR (*La Jolla*) *Molecular structures and gene organization of the acetylcholinesterases*
09:30	R.L. ROTUNDO (*Miami*) *Biogenesis of acetylcholinesterase in nerves and muscle*
10:00	W.R. RANDALL (*Miami*) *Avian acetylcholinesterase: molecular studies on the structure and regulation of the asymmetric forms in muscle*
10:20	Coffee
10:50	B.P. DOCTOR (*Washington, DC*) *Structural and immunochemical properties of fetal bovine serum acetylcholinesterase*
11:20	H. SOREQ (*Jerusalem*) *Biogenesis of human cholinesterase: from gene to protein*
11:50	P. DREYFUS (*Jerusalem*) *Structure-function relationships in cholinesterase approached by microinjection of Xenopus oocytes with clone-produced SP_6mRNA*
12:10	E. SCHMELL (*Arlington*) *Inhibition of human erythrocyte AChE by monoclonal antibodies: evidence for novel allosteric sites*
12:30	A. FUTERMAN (*Rehovot*) *Covalently attached phosphatidylinositol as a membrane anchor for acetylcholinesterase and other membrane proteins*
12:50	End of session

| 13:00 | Lunch |
| | |

PRESYNAPTIC MECHANISMS (I)

CHAIRMAN: R. RAHAMIMOFF (*Jerusalem*)

15:30	I. PARNAS (*Jerusalem*) *The calcium-voltage hypothesis for neurotransmitter release*
16:00	R.S. ZUCKER (*Berkeley*) *Presynaptic calcium domains and voltage control of transmitter release*
16:30	R. RAHAMIMOFF (*Jerusalem*) *Short term and long term modulation of neurotransmitter release*
17:00	Coffee
17:30	E. NEHER (*Gottingen*) *Calcium buffering and calcium measurement in single secretory cells under patch clamp*
18:00	P. GAGE (*Canberra*) *Neuromuscular transmission in muscles paralysed by butanedione monoxime*
18:30	End of session
18:45	Dinner

PRESYNAPTIC MECHANISMS (II)

CHAIRMAN: E. NEHER (*Gottingen*)

20:00	S.M. PARSONS (*Santa Barbara*) *Regulatory, molecular and pharmacological aspects of acetylcholine storage*
20:30	M. ISRAEL (*Gif-sur-Yvette*) *Purification of a nerve terminal protein which mediates a calcium-dependent acetylcholine release*
21:00	H. RAHAMIMOFF (*Jerusalem*) *Molecular properties of the sodium-calcium exchanger*
21:30	M.P. BLAUSTEIN (*Baltimore*) *Presynaptic potassium channels as the target sites of certain snake toxins and other drugs*
22:00	Y. YAARI (*Jerusalem*) *Presynaptic currents in vertebrate motor nerve terminals*
22:20	S.C. FROEHNER (*Hanover*) *Monoclonal antibodies to the voltage-activated calcium channel*
22.40	End of session

Wednesday, September 2nd
SIGNAL TRANSDUCTION AND MODULATION

CHAIRMAN: P. TAYLOR (*La Jolla*)

08:30   M.E. ELDEFRAWI (*Baltimore*)
Action of anticholinesterases on acetylcholine receptors

09:00   G. AMITAI (*Ness-Ziona*)
Certain muscarinic antagonists are non-competitive inhibitors of the nicotinic acetylcholine receptor

09:20   E. HELDMAN (*Ness-Ziona*)
Binding properties and transmembrane signalling in muscarinic acetylcholine receptor subtypes

09:40   K. SHERMAN (*Springfield*)
Biochemical and behavioral effects of acetylcholinesterase inhibition in brain

10:00   Coffee

10:30   E.X. ALBUQUERQUE (*Baltimore*)
Molecular basis of anticholinesterase actions on nicotinic and glutamatergic synapses: peripheral and central effects

11.00   T.S. REESE (*Woods Hole*)

11:30   Y. HENIS (*Tel-Aviv*)
Role of lipids in the regulation of rat heart muscarinic receptors and their interaction with G-proteins

11:50   Y. LASS (*Tel-Aviv*)
Neurotransmission and second messengers in Xenopus oocytes

12:20   End of session

12:30   Lunch

13:30   Excursion to Jerusalem

19:00   Dinner

ACETYLCHOLINE RECEPTOR (I)

CHAIRMAN: E. KOSOWER (*Tel-Aviv*)

20:00   A. KARLIN (*New York*)
Functional sites of the nicotinic acetylcholine receptor

20:30   J. EARNEST (*San Francisco*)
What do the structure of the acetylcholine receptor and of ion channels tell us about their function?

21:00   A. MAELICKE (*Dortmund*)
Antibodies as specific ligands of the nicotinic acetylcholine receptor

21:20   F. HUCHO (*Berlin*)

          The helix-II model of the ion channel of the nicotinic acetylcholine receptor

21:40   J.M. GERSHONI (*Rehovot*)
          Correlating structure with function in the nicotinic acetylcholine receptor

22:00   G. NAVON (*Tel-Aviv*)
          NMR studies of the binding of agonists to the acetylcholine receptor

22:20   End of session

Thursday, September 3rd

ACETYLCHOLINE RECEPTOR (II)

CHAIRMAN: F. BARRANTES (*Bahia Blanca*)

08:30   J. PATRICK (*La Jolla*)
          Expression of functional neuronal acetylcholine receptors from cDNA clones

09:00   H.A. LESTER (*Pasadena*)
          Expression of acetylcholine receptors in Xenopus oocytes

09:30   C. METHFESSEL (*Gottingen*)
          Ion conductance of genetically modified acetylcholine receptor channels

10:00   Coffee

10:30   H. BREER (*Osnabruck*)
          Receptors for acetylcholine in the nervous system of insects

10.50   G.P. HESS (*Ithaca*)
          Chemical kinetic measurements of the acetylcholine receptor with 8mv.s to ms time resolution

11.20   End of session

12:30   Lunch

ACETYLCHOLINE RECEPTOR (III)

CHAIRMAN: A. KARLIN (*New York*)

14.00   F.J. BARRANTES (*Bahia Blanca*)
          The membrane environment for the nicotinic acetyl holine receptor

14:30   M. McNAMEE (*Davis*)
          Biophysical studies of acetylcholine receptor in reconstituted membranes: role of lipids in regulating function

15.00   S. FUCHS (*Rehovot*)
The cholinergic binding site and phosphorylation sites of the nicotinic acetylcholine receptor

15.30   Coffee

16.00   R.L. HUGANIR (*New York*)
Regulation of the nicotinic acetylcholine receptor by protein phosphorylation

16:30   E.M. KOSOWER (*Tel-Aviv*)
A structural and dynamic model for the nicotinic acetylcholine receptor

17:00   End of session

17.45   Departure for closing dinner

Friday, September 4

        Departure

POSTER PRESENTATIONS

01   M. ARPAGAUS (Gif-sur-Yvette)
Transitions in molecular forms of acetylcholinesterase during the development of Drosophila melanogaster.

02   F. BACOU (Montpellier)
Acetylcholinesterase of newborn denervated fast and slow rabbit muscles: polymorphic and immunological characteristics.

03   E. BANIN (Jerusalem)
Acute administration of aluminium alters synaptic transmission.

04   M. BENNETT (Gif-sur Yvette)
Acetylcholine release from rat brain synaptosomes as measured by the chemiluminescence method.

05   C. BON (Paris)
Negatively charged phospholipids, a possible target for the presynaptic phospholipase neurotoxin from snake venoms.

06   W. FINGER (Munich)
High-frequency asynchronous release of excitatory and inhibitory transmitter quanta triggered by veratridine in nerve-muscle synapses of crayfish.

07   A. GNATT (Jerusalem)
Molecular cloning and structural characterisation of human cholinesterase genes.

08   M. JACKSON (Gottingen)
Mechanism and energetics of activation of the nicotinic receptor.

09   K. KAUFMANN (Essen)
On the role of the lipid bilayer in synaptic transmission.

10   R. LICHT (Tel Aviv)
Basal acetylcholine release from Torpedo nerve terminals

11   P. MASSON (Toulon-Naval)
Pressure effects on the carbamylation of butyrylcholinesterase.

12   J.L. MIDDLEBROOK (Frederick)
A neutralizing monoclonal antibody to crotoxin.

13   P.C. MOLENAAR (Leiden)
The effect of AH5183 on the release and storage of newly synthesized ACh in frog muscle.

14   N. MOREL (Gif-sur-Yvette)
An antiserum specific for the mediatophore, an ACh-releasing protein, exhibits presynaptic binding in Torpedo and rat neuromuscular junctions.

15   Y. MOROT-GAUDRY (Gif-sur Yvette)
Cetiedil, a drug which inhibits acetylcholine release in Torpedo marmorata electric organ.

16   S.J. MOSS (Cambridge)
Regulation of chicken muscle nicotinic acetylcholine receptor gene expression.

17 S.J. MRCHEV (*Sofia*)
Human memory modelling: Synapse level.

18 D. NEUMANN (*Rehovot*)
Cloning of the snake acetylcholine receptor &alpha.-subunit.

19 C. PRODY (*Jerusalem*)
Alternate termination sites in human cholinesterase genes.

20 R. ROSENBLUM (*Tel-Aviv*)
Coexistence of endephalins and acetylcholine in Torpedo electromotor neurons

21 A. SAFRAN (*Rehovot*)
Phosphorylation of the acetylcholine receptor.

22 TH. SCHURHOLZ (*Bielefeld*)
Reconstitution of isolated acetylcholine receptors: A critique

23 J. SKETELJ (*Ljubljana*)
Acetylcholinesterase in regenerating myotubes in vivo.

24 N. STEINBERG (*Rehovot*)
Spectroscopic characterisation of differences in the active-site conformation of aged and non-aged organophosphoryl conjugates of serine hydrolases

25 S.N. TIWARAI (*Gorakhpur*)
Physical parameters of the transformation and distribution of information

26 J.-P. TOUTANT (*Montpellier*)
Molecular forms of acetylcholinesterase in adult Drosophila: Structure and hydrophobic interactions.

27 K.W.K. TSIM (*Cambridge*)
Monoclonal antibodies to chicken acetylcholinesterase and butyrylcholinesterase.

28 M. VERDIERE-SAHUQUE (*Paris*)
Developmental aspects, cellular localization and mode of attachment of AChE in neuronal ganglionic cells.

29 H. ZAKUT (*Tel-Aviv*)
Cholinesterase synthesis in developing human oocytes examined by in situ hybridization in frozen ovarian sections.

30 R. ZAMIR (*Jerusalem*)
Chromosomal mapping of human cholinesterase genes by in situ hybridization.

31 R. ZISLING (*Rehovot*)
Antibodies to clone-produced human cholinesterase: Interaction with denatured and native cholinesterases and cross-species homologies.

ABSTRACTS

LONG-TERM EVOLUTION OF THE ACETYLCHOLINE RECEPTOR
DURING SYNAPSE FORMATION

by

Jean-Pierre CHANGEUX, André KLARSFELD, Bertrand FONTAINE
and Ralph LAUFER

Neurobiologie Moléculaire et UA CNRS 1149, Institut Pasteur,
25 rue du Dr Roux, 75725 Paris Cedex 15, France.

The acetylcholine nicotinic receptor protein (AChR) from vertebrate neuromuscular junction is subject to a long-term regulation of its distribution and properties by muscle innervation (1). In the adult synapse, the AChR is integrated into a supramacromolecular edifice which persists for weeks after denervation and involves privileged interactions with the basal lamina and/or the cytoskeleton. Its surface density (10 - 20 000 molecules per μm^2) is 100-1000 times higher than outside the synapse, it is strongly immobilized, with a long metabolic life-time ($>$ 10 days in mammals) and a short (msec) mean channel open time (not in birds). By contrast, in the non-innervated embryonic myotube, the AChR is evenly distributed at a low surface density, it is mobile, labile (half life $<$ 1 day) and its mean channel open time is long (several msec) (1).

The genesis of the adult postsynaptic domain results from a complex sequence of molecular interactions which include regulations at the transcriptional and post-transcriptional level. The evolution of the total content of AChR of a fast skeletal muscle (such as PLD in chick) comprises three main steps : 1) a rapid increase which coincides with the fusion of myoblasts into myotubes, 2) a sharp decline which corresponds to the elimination of the extra junctional AChR and, 3) a late increase, mostly postnatal, associated with the enlargement of the endplate. In the chick, the metabolic life-time of the AChR does not change throughout this evolution (except at the end of the third period) which, therefore, primarily involves regulation of AChR biosynthesis (2).

Chronic paralysis of the embryo by flaxedil interferes with step 2 which, thus, represents an electrical activity-dependent repression of AChR genes (2). This process has been analysed by the methods of molecular genetics using a chicken α-subunit genomic probe (3) in a model system consisting of chick myotubes in primary culture. Blocking their spontaneous electrical activity by tetrodotoxin (TTX) causes,

after 2 days, a 13-fold increase of α-subunit mRNA level (against an
only 2-fold increase of surface AChR) while α-actin mRNA levels do not
change. Denervation of chick leg muscle gives similar results. Southern
blot data are consistent with the presence in the chicken of a unique
chromosomic gene encoding the α-subunit, whose expression would be
differentially regulated during development. The 5' end and part of the
upstream flanking region of this gene was isolated and sequenced (4).
Transcription initiates at the same position in innervated and
denervated muscles (see also 5). TATA and CAAT boxes and a potential
Spl binding site are found upstream. This α-subunit promoter, including
850 bp of the 5' flanking sequence, was inserted into a plasmid vector
in front of a chloramphenical acetyltransferase (CAT) gene (4). This
construct directed high CAT expression in transfected mouse C2.7
myotubes but not in unfused C2.7 myoblasts or non myogenic mouse 3T6
cells. It thus contains DNA sequences important for tissue specificity
and developmental regulation (step 1). Yet, it is not known whether
these sequences contain the target for the electrical activity-
dependent regulation of α-subunit gene expression.

The maintenance and late increase in number (step 3) of AChR at the
level of the endplate while extrajunctional AChR disappears requires
the intervention of an anterograde signal from neural origin.
Calcitonin-gene-related peptide (CGRP), a peptide shown to coexist with
acetylcholine in chick spinal cord motoneurons, increases surface and
total AChR by about 50 % in cultured chick myotubes without affecting
AChR turnover or total protein synthesis (6). This increase was
additive with that elicited by TTX, but not by cholera toxin which
activates adenylate cyclase. Parallel results were obtained when AChR
α-subunit mRNA was quantitated by Northern blots (7). CGRP increases
the cAMP content of myotubes and stimulates membrane-bound adenylate
cyclase in the range of concentration where it enhances AChR α-subunit
gene expression (8). The phorbol ester TPA abolishes the increase of
α-subunit mRNA caused by TTX but not by CGRP suggesting that distinct
second messengers are involved in the regulation of AChR biosynthesis
by electrical activity and by CGRP.

The data are interpreted in terms of a model (9) which assumes that :
1) in the adult muscle fiber, nuclei may exist in different stages of
gene expression in subneural and extra junctional areas (see also 10),
2) different second messengers elicited by neural factors or electrical
activity regulate the state of transcription of these nuclei via
trans-acting allosteric proteins. Distinct families of genes involved
in the functional organisation and maintenance of the endplate

(including the several AChR subunits (11)), may be concerned by this regulation. In the case of the AChR αβγ and δ subunits the relevant genes are distributed, in the mouse, on three different chromosomes (α Chr 17, β Chr 11 and γ and δ Chr 1) (12) and are thus regulated by trans-activating factors.

The subneural post-transcriptional clustering and metabolic stabilisation of the AChR which takes place at the level of the developing endplate involves, in addition, a complex sequence of molecular interactions with, in particular, the basal lamina and/or cytoskeletal proteins. The role of the 43 000 v_1 protein (13), which specifically binds to the cytoplasmic face of the AChR, is discussed in this framework and its interaction with the cytoskeleton demonstrated (14). Speculations about the plausible extension of some of these views to the selective stabilisation of neuronal synapses are presented (15).

REFERENCES :

(1) Changeux, J. P. (1981) The acetylcholine receptor : An "allosteric" membrane
protein. In "Harvey Lectures". Acad. Press Inc. 75, 85-254.

Salpeter, M. and Loring, R.H.(1986) Nicotinic acetylcholine receptors in
vertebrate muscle : properties, distribution and neural control. Progr.
Neurobiol. 25, 297-325.

Merlie, J.P. and Smith, M.M. (1986) Synthesis and assembly of acetylcholine
receptor, a multisubunit membrane glycoprotein. J. Memb. Biol. 91, 1-10.

(2) Betz, H., Bourgeois, J.P. and Changeux, J.P. (1980) Evolution of cholinergic
proteins in developing slow and fast skeletal muscles from chick embryo. J.
Physiol. 302, 197-218.

Burden, S. (1977a) Development of the neuromuscular junction in the chick
embryo. The number, distribution and stability of the acetylcholine
receptor. Dev. Biol. 57, 317-329.

Burden, S. (1977b) Acetylcholine receptors at the neuromuscular junction :
Developmental change in receptor turnover. Dev. Bio 61, 79-85.

(3) Klarsfeld, A. and Changeux, J.P. (1985) Activity regulates the level of
acetylcholine receptor alpha-subunit mRNA in cultured chick myotubes. Proc.
Natl. Acad. Sci. USA, 82, 4558-4562.

(4) Klarsfeld, A., Daubas, P., Bourachot, B. and Changeux, J.P. (1987) A 5'
flanking region of the chicken acetylcholine receptor alpha-subunit gene
confers tissue-specificity and developmental control of expression in
transfected cells. Mol. Cell Biol. (in press).

(5) Buonanno, A. and Merlie, J.P. (1986) Transcriptional regulations of
nicotinic acetylcholine receptor genes during muscle development. J. Biol.
Chem. 261, 11452-11455.

(6) Fontaine, B., Klarsfeld, A., Hökfelt, T. and Changeux, J.P. (1986)
Calcitonin gene-related peptide, a peptide present in spinal cord
motoneurons, increases in number of acetylcholine receptors in primary
cultures of chick embryo myotubes. Neuroscience Letters 71, 59-65.

(7) Fontaine, B., Klarsfeld, A. and Changeux, J.P. (1987) Calcitonin-gene
related peptide and muscle activity regulate acetylcholine receptor
α-subunit mRNA levels by distinct intracellular pathways. J. Cell Biol. (in
press).

(8) Laufer, R. and Changeux, J.P. (1987) Calcitonin gene-related peptide
elevates cyclic AMP levels in chick skeletal muscle : possible neurotrophic
role for a coexisting neuronal messenger. EMBO J. (in press).

(9) Changeux, J.P., Klarsfeld, A. and Heidmann, T. (1986b) The acetylcholine
receptor and molecular models for short and long term learning. Dahlem
Konferenzen. In "The cellular and molecular bases of learning". J.P.
Changeux and M. Konishi, ed. Wiley London p. 31-83.

(10) Merlie, J. and Sanes, J.R. (1985) Concentration of acetylcholine receptor
mRNA in synaptic regions of adult muscle fibers. Nature 317, 66-68.

(11) Takai, T., Noda, M., Mischina, M., Shimizu, S., Furutani, Y., Kayano, T.T.,
Ikeda, T., Kubo, T., Takahashi, H., Takahashi, T., Juno, M. and Numa, S.
(1985) Cloning, sequencing and expression of cDNA for a novel subunit of
acetylcholine receptor from calf muscle. Nature 315, 761-764.

Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S.,
Methfessel, C. and Sakmann, B. (1986) Molecular distinction between fetal
and adult forms of muscle acetylcholine receptor. Nature 321, 406-411.

(12) Heidmann, O., Buonanno, A, Geoffroy, B., Robert, B., Guénet, J.L.,
Merlie, J.P. and Changeux, J.P. (1986) Chromosomal localization of muscle
nicotinic acetylcholine receptor genes in the mouse. Science 234, 866-868.

(13) Sobel, A., Weber, M. and Changeux, J.P. (1977) Large scale purification of
the acetylcholine receptor protein in its membrane-bound and detergent
extracted forms from Torpedo marmorata electric organ. Eur. J. Biochem. 80,
215-224.

Cartaud, J., Sobel, A., Rousselet, A., Davaux, P. F. and Changeux, J. P.
(1981) Consequences of alkaline treatment for the ultrastructure of the
acetylcholine-receptor-rich membranes from Torpedo marmorata electric
organ. J. Cell. Biol. 90, 418-426.

Nghiêm, H .O., Cartaud, J., Dubreuil, C., Kordeli, C., Buttin, G. and
Changeux, J. P. (1983) Production and characterization of a monoclonal
antibody directed against the 43,000 M.W. nuy polypeptide from Torpedo
marmorata electric organ. Proc. Natl. Acad. Sci. USA 80, 6403-6407.

Burden, S.J. (1985) The subsynaptic 43KD protein is concentrated at
developing nerve-musle synapses in vitro. Proc. Natl. Acad. Sci. U.S.A. 82,
7805-7809.

Froehner, S. C., Gulbrandsen, V., Hyman, C., Jeng, A. Y., Neubig, R.R. and
Cohen, J. B. (1981) Immunofluorescence localization at the mammalian
neuromuscular junction of the M_r 43,000 protein of Torpedo postsynaptic
Membranes. Proc. Natl. Acad. Sci. USA 78, 5230-5234.

(14) Kordeli, E., Cartaud, J., Nghiêm, H.O., Pradel, L.A., Dubreuil, C., Paulin,
     C. and Changeux, J.P. (1986) Evidence for a polarity in the distribution of
     proteins from the cytoskeleton in Torpedo marmorata electrocytes. J. Cell
     Biol. 102, 748-761.

(15) Changeux, J.P. (1986) Coexistence of neuronal messengers and molecular
     selection. Progr. Brain Res. 68, 373-403.

MULTIPLE SITES AND MODES FOR THE REGULATION
OF GROWTH OF CHOLINERGIC AXONS.

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Synapses can be modified by remodelling their structure. One major way of doing so is by inducing the innervating neuron to sprout and form additional synaptic connections with its target. Our experimental findings in the motor system of amphibia and mammals suggest that mechanisms regulating sprouting and synapse formation can be divided into three major categories: peripheral, central and transneuronal. We define: (1) peripheral mechanisms, those that involve a direct growth promoting effect of factor(s) on the peripheral extensions of the motoneuron that responds with sprouting, (2) central mechanisms, those that involve the regulation of axonal growth at the level of the motoneurons' cell body, and (3) transneuronal mechanisms, those involving the transfer of a signal for growth from one neuron to another within the spinal cord.

We examined the pattern of innervation to individual muscle fibers of cutaneous-pectoris (CP) and sartorius (S) muscles of the frog, and of peroneal (P) and extensor digitorum longus (EDL) muscles of the mouse. The morphology of neuromuscular junctions was studied by light microscopy in silver-cholinesterase (CP, S, P, EDL) and zinc iodide stained muscles (CP), and by electron microscopy (CP). Silver-cholinesterase preparations revealed some degree of sprouting and synapse formation in all intact muscles of normal animals. Sprouting and synapse formation were enhanced in intact muscles by severing nerves innervating contralateral homologous muscles.

We hypothesize a transneuronal mechanism to underlie the induction of sprouting following contralateral axotomy. The mechanism formulates that axotomy initiates a signal for growth in the cell bodies of severed motoneurons. The signal is then transferred transneuronally across the spinal cord to intact contralateral motoneurons that respond with sprouting. The experimental results that led to the formulation of the transneuronal mechanism for the induction of sprouting and synapse formation (SSF) did so by ruling out alternative mechanisms, and by being in accord with predictions of the suggested mechanism. Alternatives to the transneuronal mechanism are that sprouting was induced by products of degeneration and denervation or by inactivity of denervated muscles. This was ruled out by experiments demonstrating: (1) contralateral axotomy was followed by SSF only when the severed and sprouting motoneurons were located in the same segments of the spinal cord (CP, S, P, EDL), thus ruling out the role of systemic distribution of degeneration and denervation products; (2) SSF developed in CP muscles after removing contralateral muscles, thus ruling out the necessity for the presence of degeneration and denervation products; (3) contralateral axotomies that were placed in too close proximity to the spinal cord failed to induce sprouting (CP), thus ruling out the role of inactivity of denervated muscles and the role of degeneration and denervation products. Predictions of the transneuronal mechanism that were met are: (1) the time to onset of SSF is shortened when the contralateral axotomy is placed closer to the spinal cord (CP, P, EDL). (2) Axotomized regenerating and intact contralateral

sprouting motoneurons demonstrate an increase in the incorporation of uridine into RNA following unilateral axotomy (CP).

We further suggest a _central_ mechanism for the induction of sprouting. We hypothesize a role for a muscle derived trophic substance as a regulator of axonal growth; the site of regulation being the motoneurons' cell body. This proposition is based on the following experimental data: (1) SSF followed the blockade of retrograde axonal transport in contralateral nerves (CP); (2) SSF was induced in both ipsilateral and contralateral motoneurons after depriving the ipsilateral neurons of their target muscle fibers (CP). The common denominator of axotomy, the blockade of the retrograde axonal transport, and the removal of muscle fibers is the prevention of the retrograde supply of a muscle derived trophic substance.

Direct evidence for the existence of _peripheral_ mechanisms for the induction of SSF is provided in a series of experiments in which motor axons that were isolated from their cell bodies responded with SSF after exposing them to minimal doses of colchicine (CP).

DEATH OF NEURONS AS A CONSEQUENCE OF EXCESS INTRACELLULAR TRANSMITTER?

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Motoneurons of the dorsal motor vagal nucleus (DMVN) of the adult guinea pig disappear slowly following cervical vagotomy, with a time constant of 8.6 months (Laiwand, Werman & Yarom, J. Comp. Neurol. 256: 527-37, 1987). Following vagotomy, the motoneurons become more excitable, apparently as a result of marked decreases in Ca^+-activated K^+ conductance and in the voltage dependent transient current $I_{K,A}$.

Acetylcholinesterase activity in these neurons decreases markedly following vagotomy, suggesting that ACh may reach higher than normal concentrations. In fact, ACh injected into normal vagal motoneurons acutely blocks various K^+ conductances, particularly the Ca^{2+}-activated K^+ conductance (Yarom, Bracha & Werman, Neuroscience 16: 739-52, 1985). These actions of ACh are not produced by extracellular application but are reproduced and enhanced by intracellular injection of blockers of acetylcholinesterase activity. Thus it is possible that a major part of the physiological changes produced by axotomy are reproduced by excess intracellular ACh in a cholinergic neuron.

In fact, the vagal motoneuron only appears to be more vulnerable following vagotomy. An acute process, lasting about 25 days and leading to cell disintegration, appears to be superimposed on the chronic vulnerability. Reduction of K^+ conductances which serve to inhibit firing leads to increased neuronal firing and probably to increases in internal Ca^{2+} concentrations, which may be the acute factor in cell death.

It is of interest that non-cholinergic neurons such as the Purkinje cell revert to a primitive state following axotomy and express latent enzymes related to ACh metabolism.

THE APPEARANCE OF TWO DISTINCT TYPES OF SUBSYNAPTIC SPECIALIZATIONS WITH AChE ACTIVITY IN REGENERATING RAT MUSCLE

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One of the basic postulated requirements of the humoral theory of cholinergic transmission is the presence of AChE activity at the postsynaptic membrane, (in the vicinity of the cholinoreceptor). Nevertheless, the activity and the distribution of AChE at different peripheral and central cholinergic synapses varies considerably, ranging from the extremely high activity in the synaptic clefts of motor endplates, to the neuronal synapses of caudate nucleus and sympathetic ganglia, where the accumulation of AChE is much less dense and its distribution appears predominantly extrajunctional. Various tissues with cholinergic synapses differ also in regard to the regulating effect of nerve on the level and distribution of AChE activity and the pattern of its molecular forms.

In skeletal muscle the main target susceptible to various regulatory influences on AChE seems to be the muscle cell itself. During ontogenesis AChE appears in early myoblasts and attains a high activity before innervation. Following the formation of neuromuscular junctions, AChE, previously distributed along the muscle cell begins to accumulate at the junction. Concomitantly the sarcolemmal specialization is beeing formed and a gradual change of AChE molecular forms is taking place untill maturation is reached.

The ability of muscle cells to focally accumulate AChE and to maintain junctional specialization of sarcolemma remains after denervation. Furthermore, under in vivo aneural conditions, muscle fibers regenerating from myoblasts within degeneration-resistant basal laminae accumulate newly synthesized AChE at the sites of previous motor endplates. In addition, along regenerating myotubes, extrajunctional AChE accumulations and postjunctional specializations are beeing formed. Pretreatment of muscles with papain before reinplantation prevents the formation of both types of accumulations, without affecting muscle regeneration.

It is suggested that basal junctional lamina stores a protein or a protein-bound substances which triggers in aneurally developing myotubes the sarcolemmal altera-tion and focalization of AChE.

AGRIN: AN EXTRACELLULAR SYNAPTIC ORGANIZING MOLECULE. Uel J. McMahan.
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Studies conducted in this laboratory have demonstrated that the portion
of a muscle fiber's basal lamina sheath that occupies the synaptic cleft at
the neuromuscular junction has stably bound to it molecules that direct the
formation of postsynaptic apparatus on regenerating muscle fibers.
Accordingly, if muscles are damaged in ways that spare the basal lamina
sheaths of the muscle fibers, the new muscle fibers that develop within the
sheaths form aggregates of acetylcholine receptors (AChR) and acetyl-
cholinesterase (AChE) where they contact the synaptic sites on the sheaths
despite the absence of axon terminals. The extracellular synaptic organizing
molecules in the synaptic basal lamina may be identical to those molecules
that mediate the nerve-induced formation of AChR and AChE aggregates in
developing myofibers during synaptiogenesis in the embryo and they may also be
involved in the maintenance of the postsynaptic apparatus in the adult.

Our studies have also led to the identification of agrin, a protein that
is extracted from the synapse-rich electric organ of Torpedo californica and
that may be similar to the AChR- and AChE-aggregating molecules in the basal
lamina at the neuromuscular junction. For example, agrin is found in basal
lamina containing fractions of the electric organ, it induces the formation of
patches in cultured myotubes that contain a high concentration of AChRs, AChE
and other components of the postsynaptic apparatus, low levels of a similar
factor are found in extracts of muscle, and monoclonal antibodies directed
against agrin recognize molecules highly concentrated in the synaptic basal
lamina at the neuromuscular junction in vivo. We have now purified agrin to
homogeneity, determined its n-terminal amino acid sequence, and prepared
oligonucleotide probes with the aim of using molecular genetic techniques to
characterize agrin and study how the expression of agrin is regulated during
development and regeneration.

I will document the above findings, discuss our progress in characteriz-
ing agrin and present results showing that the cell bodies of motor neurons
contain agrin-like molecules, consistent with the hypothesis that the agrin-
like molecules in the synaptic basal lamina are produced by motor neurons and
released by their axon terminals.

AGRIN: MECHANISM OF ACTION. Bruce G. Wallace. Dept. of Neurobiology, Stanford University School of Medicine, Stanford, CA. 94305.

Agrin induces patches on chick myotubes in culture at which at least six components of the postsynaptic apparatus are concentrated: two extracellular matrix molecules (a heparin sulfate proteoglycan and the A_{12} asymmetric form of acetylcholinesterase [AChE]), three membrane proteins (acetylcholine receptors [AChRs] and globular forms of AChE and butyrylcholinesterase), and a cytoplasmic protein (a 43 kD receptor-associated protein). All of the aggregating activities are immunoprecipitated by each of 5 different anti-agrin monoclonal antibodies and all activities copurify and comigrate on gel filtration columns. The studies reported here were aimed at characterizing agrin's effects on AChR distribution and metabolism as a step toward determining agrin's mechanism of action and comparing it to the mechanism of nerve-induced receptor aggregation at developing neuromuscular junctions.

The accumulation of AChRs at developing neuromuscular junctions begins with the formation of a loose aggregate of small clusters of AChRs that subsequently coalesce into a large, relatively uniform patch. When agrin was added to the medium bathing chick myotubes small (<4µm²) aggregates of AChRs began to appear within 2 hrs and increased rapidly in number until 4 hrs. Over the next 12-20 hrs the number of aggregates per myotube decreased as the mean size of each aggregate increased to ≈15 µm². If newly formed junctions in vivo are denervated there is a marked dispersal of junctional AChRs. Similarly, in agrin-treated myotubes in culture, aggregates of AChRs remained as long as agrin was present in the medium; if agrin was removed the number of aggregates declined slowly. Most AChRs accumulated into agrin-induced aggregates by lateral migration, as do many of the AChRs that aggregate at developing neuromuscular junctions. Agrin did not alter the rate of appearance of new AChRs.

AChRs on embryonic myofibers are degraded with a $t_{1/2}$≈1 day. Days to weeks after AChRs accumulate at neuromuscular junctions their rate of degradation slows such that AChRs aggregated at adult neuromuscular junctions are degraded with a $t_{1/2}$≈10 days. In our chick myotube cultures, ≈85% of the AChRs were degraded at a rapid rate ($t_{1/2}$≈1d), ≈15% turned over more slowly ($t_{1/2}$≈10d). Agrin-containing extracts increased the proportion of slowly turning over receptors to ≈20%.

The formation and maintenance of AChR aggregates at developing neuromuscular junctions requires Ca^{++}. Agrin-induced AChR aggregation also required Ca^{++}; a half-maximal response occurred at 0.2 mM Ca^{++}. Typical inorganic Ca^{++} antagonists, such as Co^{++}, Mn^{++}, and Ni^{++}, inhibited agrin-induced AChR aggregation. Two other divalent cations, Mg^{++} and Sr^{++}, did not inhibit receptor aggregation in the presence of Ca^{++}, but could not substitute for Ca^{++}. Agrin-induced receptor aggregation also was inhibited by the phorbol ester TPA, an activator of protein kinase C; 20 nM TPA completely prevented AChR aggregation. Two dimensional SDS-PAGE of extracts of cultures identified several polypeptides that were phosphorylated in response to TPA treatment. Agrin itself did not change the pattern of phosphoproteins.

Thus agrin induces AChRs in the myotube plasma membrane to accumulate into aggregates by a rapid, Ca^{++} dependent process that is inhibited by protein kinase C-mediated protein phosphorylation and is accompanied by a decrease in the rate of degradation of a fraction of AChRs. In many ways agrin's effects on cultured myotubes mimic events that occur during formation of the neuromuscular junction in vivo.

ACETYLCHOLINESTERASE IN REGENERATING NEUROMUSCULAR JUNCTIONS.

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Acetylcholinesterase (AChE) in skeletal muscle is concentrated at the neuromuscular junctions. If muscles are damaged in ways that spare the myofiber basal lamina sheaths, new myofibers develop within the sheaths and the damaged axons regrow to form new neuromuscular junctions on the regenerating myofibers at the original synaptic sites. AChE accumulates at the regenerating neuromuscular junctions, and as at normal ones, a substantial fraction of the enzyme is found in the synaptic cleft where it is associated with the synaptic portion of the basal lamina, passing between nerve terminal and myofiber. The studies we describe were aimed at learning whether synaptic AChE is produced by muscle, nerve or both and whether basal lamina directs its accumulation at the synaptic sites.

Frog muscles were damaged in a way that caused disintegration of the myofibers while sparing basal lamina sheaths. Myofibers were allowed to regenerate but reinnervation was deliberately prevented. The regenerating myofibers produced new AChE which preferentially accumulated at points where the plasma membrane of the new muscle fibers was apposed to the region of the basal lamina that had occupied the synaptic sites at the original neuromuscular junctions. The newly formed enzyme became incorporated into the synaptic basal lamina. These results demonstrate that regenerating myofibers produce synaptic AChE and that its accumulation is directed by synaptic basal lamina.

A complementary study was carried out on operated frog muscles in which myofibers had been removed from the basal lamina sheaths, while leaving intact motor axons, nerve terminals and synaptic basal lamina sheaths. The nerve terminals persisted at the synaptic sites on the sheaths in the absence of myofibers and had all the structural features of normal terminals. We found out that these terminals produced surface AChE and a substantial amount of the newly formed enzyme became associated with the extracellular matrix at the synaptic site, primarily with the synaptic basal lamina adjacent to the terminal. Nerve terminals that reinnervated synaptic basal lamina behaved similarly. This finding shows that some of the AChE that is made and transported by the motor nerve contributes _directly_ to the synaptic cleft enzyme.

We conclude that myofibers and nerve terminals can produce synaptic cleft AChE and that its appearance in the regenerating neuromuscular junction is directed by synaptic basal lamina. Other factors such as nerve and muscle activity may play a role in regulating the production and maintenance of the synaptic enzyme.

ROLE OF A NEUROTROPHIC FACTOR AND CALCIUM CHANNELS IN THE
REGULATION OF ACETYLCHOLINESTERASE IN CULTURED MUSCLE

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The accumulation of AChE in C_2 cultured mouse muscle cells
was found to be stimulated by incubation of the cultures with
extracts prepared from rat brains. We have purified the stim-
ulatory activity by various HPLC procedures. The neurotrophic
factor (NTF) preparation increased the accumulation of true
AChE (EC 3.1.1.7) in a dose dependent manner. Incubation with
NTF increased all AChE molecular forms in the muscle cultured
cells. However, the increase of the 16S form was relatively
more pronounced. In addition, treatment with NTF changed the
cellular distribution of AChE and increased the number of mem-
brane patches containing high concentrations of enzyme.
Increasing Ca^{2+} concentration in the culture medium synergis-
tically increased the effect of NTF on AChE accumulation,
while Ca^{2+} by itself had only a margine effect. The voltage
dependent Ca^{2+} channel blocker, nitrendipine, inhibited the
effects of NTF on the accumulation of AChE. It also reduced
the amount of AChE in the untreated cells although to a lesser
extent. Nitrendipine also inhibited the clustering of AChE
seen in NTF-treated cells. Moreover, preincubation of C_2 mus-
cle with NTF increased the $^{45}Ca^{2+}$ rate of influx into the
cells compared with control.
The results, thus, suggest that NTF increases the uptake of
Ca^{2+} into the cells, possibly through nitrendipine sensitive
sites. This uptake of Ca^{2+} plays a role in the regulation of
AChE in the cells.

CHANGES IN THE LEVEL OF ACETYLCHOLINE RECEPTORS ARE MEDIATED BY CALCIUM
CONCENTRATION IN THE SARCOPLASMIC RETICULUM

by Shainberg, A., Freud-Silverberg M. and Brik, H.
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Regulation of the AChR level, assayed by $^{125}I-\alpha$-bungarotoxin
($^{125}I-\alpha$-Bgt) binding sites was studied in chick skeletal muscles diff-
erentiated in vitro. A large variety of agents known to affect muscle con-
tractions and calcium movement were investigated. The level of AChR is in-
creased by various treatments which have a similar effect on cytoplasmic
Ca^{2+}, presumably its accumulation in the sarcoplasmic reticulum (SR).
Thus, tetrodotoxin (TTX) sodium dantrolene, or Ca channel blockers like
D600, which inhibit the spontaneous contractions and therefore probably
cause accumulation of Ca^{2+} in the SR, enhanced receptors synthesis.
Muscle inactivity per se is not enough to cause receptor elevation. When
TTX was given with low Ca^{2+} (50 uM), there was no increase in receptor's
level. In contrast, there was receptor elevation when immobilization was
achieved with D600 in low Ca^{2+}. These results indicate that Ca^{2+} is
required for the induction process of the receptors synthesis. However,
it does not have to be from an external source. The apparent contradic-
tion between the effects of low Ca^{2+} in TTX and those of D600 on AChR,
could be explained by the possibility that in the TTX group Ca is not
accumulated in the SR. According to this model, redistribution of Ca^{2+}
in the opposite direction - from the SR to the myoplasm - is expected to
bring about a decrease in the level of AChR. Caffeine, which causes
Ca^{2+} release from the SR, indeed decreased the $^{125}I-\alpha$-Bgt binding.
Comparable effects on Ca distribution and the level of AChR were observed
using electrical stimulation (ES), carbamylcholine or ryanodine.
Electrical stimulation of the muscle cultures in a frequency of 100 Hz for
1s was more efficient in reducing the level of the receptors than chronic
1 Hz or 10 Hz for 10 s each 100 s. Exposure of the myotubes to ES in high
K, which paralyzed the muscle, also reduced the level of the receptors.
Furthermore, ES in low voltage, insufficient to elicit muscle contraction,
was also effective in reducing the level of the receptors. We conclude that
intracellular Ca^{2+} release from the SR is a necessary mediator for the
decline in AChR synthesis. Since even media taken from electrically stimu-
lated muscle cultures are capable of inhibiting receptor synthesis a prop-
erty lost by heating, we assume that electrically stimulated myotubes prob-
ably release to the medium a protein(s) responsible for reducing AChR syn-
thesis. The level of this protein is probably regulated by Ca content in
the SR. Calcium in high concentration binds this protein and the synthesis
of AChR ensues. In conditions of low Ca in the SR this protein remains
free, and synthesis of receptors is reduced.

THE EARLY MOMENTS OF SYNAPTOGENESIS: A PHYSICO-CHEMICAL VIEW.

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Synaptogenesis depends upon the interactions between specific molecules. Molecular specificity, however, operates only when the interacting molecules are brought within molecular ranges. Nonspecific physical forces are probably responsible for the spatial arrangement of molecules that sets the stage for specific molecular interactions. Experimental and theoretical evidence suggest that an intricate combination of specific chemical interactions and nonspecific physical processes may occur during the early moments of synaptogesis between nerve and muscle cells. Two cases will be considered:

1. DIFFUSION-MEDIATED TRAPPING of specific membrane-bound molecules at the site of nerve-muscle contact may be an integral part of the process of cell-cell recognition, the development of selective cell adhesion, and the selective synaptic localization of transmitter receptors and ion channels. The absence of basal lamina during the early phase of synaptogenesis allows for the close apposition of pre- and post-synaptic plasma membranes. Thus, direct binding of membrane components of two contacting surfaces is feasible. This binding provides the mechanism for the diffusion-mediated trapping of specific molecules.

2. ELETROKINETIC MIGRATION of membrane and cytoplasmic components induced by local synaptic currents may impose a bias in their distribution, and facilitate localized molecular interactions at the synaptic site. Contact with the muscle cell triggers a pulsatile release of acetylcholine from the nerve terminal, leading to local synaptic current within seconds after nerve-muscle contact. Such current generates substantial electric field (0.1 to 1.0 V/cm) in the cytoplasm and along the membrane of the muscle cell. Studies on model systems indicate that such a field is capable of inducing electrokinetic (electrophoretic or electro-osmotic) migration of both cytoplasmic and membrane components. The direct electrokinetic action of the synaptic current may help to localize or concentrate the cytoplasmic second messengers, the cellular organelles, and the substrates for local molecular interactions. It also provides a cellular mechanism for an activity-dependent stabilization of the synapse, and a basis for competition among multiple synaptic contacts on the same postsynaptic cell.

SHORT AND LONG TERM EFFECTS OF ALUMINUM ON SYNAPTIC TRANSMISSION AND ON THE
ACTIVITY OF CULTURED NERVE CELLS. Halina Meiri, Eyal Banin and Michal Roll,
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Elevated brain concentrations of aluminum which are toxic to many
biochemical processes are found in a few neurological conditions in humans.
In the senile and presenile dementia of Alzheimer type and in the Guam
parkinsonian-dementia complex, aluminum is localized at sites bearing typical
neurofibrillary degenerations. In dialysis encephalopathy extremely high
levels of aluminum in the brain tissue occur in the absence of characteristic
neuropathology. The etiological importance of aluminum to these diseases is
still questionable and its contribution to the neurological symptoms in human
patients is difficult to evaluate. However, in experimental animals
neurological disorders associated with neurofibrillary degeneration have been
induced by intracerebral injection with aluminum.

In this study, we attempted to relate to functional rather than
pathological aspects of aluminum neurotoxicity at the level of a single nerve
cell and an individual synapse. Electrical activity of two isolated
preparations exposed to aluminum was examined: the frog cutaneous pectoris
motor synapse and the tumor cells of the mouse neuroblastoma line N1E-115.
Conventional electrophysiological techniques and computer assisted analysis
were utilized to monitor synaptic potentials and neuronal action potentials at
aluminum exposed cells.

The following results were observed:
A. Isolated frog neuromuscular junction:
Aluminum (6-200μg/ml) has a dual effect on synaptic transmission:
1. Within two hours of aluminum administration to the experimental bath
both the quantal content of evoked acetylcholine release and the
frequency of spontaneous transmitter secretion are simultaneously
augmented by (15-100%) in a dose dependent manner. This effect is
independent of the concentration of calcium ions in the extracellular
solution.

2. Deterioration of activity at nerve terminals detached from their motor
nerve cells is accelerated in the presence of aluminum. An isolated
control neuromuscular preparation constantly stimulated at a frequency of
0.1-0.3 Hz, stops releasing transmitter after 18.0+2.4 hours. In the
presence of aluminum evoked acetylcholine release stops earlier, after
11.2+3.2 hours. Spontaneous secretion of individual quanta from an
unstimulated nerve persists for more than 36 hours independently of
aluminum.

B. Neuroblastoma cells in culture:

Differentiated neuroblastoma cells in culture deteriorate when exposed to
aluminum. Following addition of $AlCl_3$ to the culture dish containing the
cells, progressive changes in the shape of their action potential are
observed. Within 4-6 days, the cells fail to respond to electrical
stimulation. The vulnerability to aluminum toxicity is higher in
differentiated cells than during the other phases of the cell cycle.

Proliferating neuroblastoma cells maintained in aluminum containing
solution differentiate normally when exposed to DMSO; they develop
neurites and respond to electrical stimulation with characteristic
polyphasic action potentials. However, once differentiated they
deteriorate within 4-6 days whereas control cells, kept without aluminum,
function properly for at least 14 days.

In conclusion, aluminum at concentrations found in the brain tissue of
some demented patients affects the activity of isolated nerve cells and
synapses: it interferes with the control of synaptic transmission and
accelerates the deterioration of mature nerve cells.

ANTIBODIES TO CHOLINERGIC NEURONS IN ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is associated with a reduction in presynaptic cholinergic parameters in the cortex and hippocampus. Although the etiology and pathogenesis of AD are not known, several reports indicate the involvement of immunological mechanisms. In the present work we examined the existence of antibodies in AD sera wnicn bind specifically to cholinergic neurons and investigated their role in the disease. As antigens we employed the purely cholinergic electromotor neurons of the electric fisn Torpedo. These neurons are chemically homoyeneous and cross react antigenically with human and mammalian cholinergic neurons.

Our findings, based on immunoblot assays, show that AD immunoglobulins bind to a specific polypeptide (PK120) in the cholinergic cell bodies of Torpedo electromotor neurons, and that immunoglobulins of patients with other dementias and neurological disorders do not bind to the tnis antigen.

The possibility that the anti-PK120 antibodies play a role in the development of cholinergic dysfunction in AD was examined by immunizing rats with Torpedo cholinergic cell bodies and assessing the resulting effects on their brain cholinergic neurons and on their behaviour. Prolonged immunization with this antigen (>1 year) resulted in impaired spatial learning (T-test and Morris water maze) and in specific changes in the level of cholineacetyltransferase and acetylcnolinesterase activities in the rat brain. The extent to whicn this system may be used as a model for the study of immunological mecnanisms in AD will be discussed.

Alternative splicing generates different mRNAs for the catalytic subunits of globular and asymmetric forms of Torpedo acetylcholinesterase

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The electric organs of Torpedo contain asymmetric and globular forms of
AChE. In the asymmetric forms, the catalytic subunits (type A) are associated
through disulfide bonds to a collagen-like tail, which inserts the enzyme in the
basal lamina. In the globular, dimeric form, the catalytic subunits (type G)
possess a C-terminal-linked glycolipid which anchors them in the cellular
membranes.

We have used cDNA probes corresponding to the C-terminal portion of the A
subunit to demonstrate the existence of distinct mRNAs by S1 mapping analysis,
and thus determined the position of a divergence. We have isolated a cDNA clone,
the sequence of which differs from that of the A form (Schumacher et al., 1986;
Sikorav et al., 1987), downstream from that divergence. The divergent sequence
codes for a 30 aminoacid extension possessing the following characteristics.
1) It starts with Ala-Cys (at positions 538-539 of the mature protein), i.e. the
sequence of the C-terminal peptide which is amide-linked to the glycolipid, as
determined by P. Taylor (personal communication). 2) The extension showed a clear
homology to the corresponding sequence of Drosophila AChE, which is indeed known
to consist of glycolipid-anchored dimers. 3) The end of the 30 aminoacid
extension is hydrophobic.

We conclude that this cDNA corresponds to the catalytic subunit of the
amphiphilic G_2 form. Like other glycolipid anchored proteins, this type of
AChE subunit is therefore synthesized as a precursor possessing a C-terminal
extension which is exchanged for the glycolipid after completion of its
synthesis.

Further analyses of cDNA and genomic sequences will clarify the
mechanisms of alternative splicing in the generation of AChE molecular forms.

Schumacher, M., Camp, S., Maulet, Y., Newton, M., McPhee-Quigley, K., Taylor,
S.S., Friedmann, T. and Taylor, P. (1986) Nature, 319, 407-409.
Sikorav, J.-L., Krejci, E. and Massoulié, J. (1987) EMBO J., 6, 1865-1873.

**Molecular Structures and Gene Organization of the
Cholinesterase Species.** Palmer Taylor, Mark
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The determination of the primary structure of the
cholinesterases obtained either from the c-DNA
sequence or from direct amino acid sequencing has
yielded essential information on structures of the
various cholinesterases and their relationship to
other proteins. Analysis of disulfide-linked
peptides has shown three internal loops in the
molecule and that the most C-terminal cysteine
forms the intersubunit disulfide linkage. Torpedo
acetylcholinesterase and human butyrylcholin-
esterase show 53% residue identity. Less homology
is seen with the Drosophila enzyme but the cysteine
positions show similar disulfide arrangements in
the enzyme. The overall sequence homology between
acetylcholinesterase, the C-terminal portion of
thyroglobulin, an inducible protein in Dictyoste-
lium and other esterases suggests a larger gene
family for acetylcholinesterase. The availability
of several c-DNA clones for acetylcholinesterase
also permits an analysis of messenger RNA species.
Genomic cloning and RNAase digestion experiments
reveal a point of divergence in the acetylcholin-
esterase messages and have permitted the analysis
of the molecular basis of the polymorphism of
acetylcholinesterase. This information has been
correlated with differences in amino acid sequence
between the asymmetric and hydrophobic species of
acetylcholinesterase.

THE BIOGENESIS OF ACETYLCHOLINESTERASE IN NERVES AND MUSCLE

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Acetylcholinesterase (AChE) is expressed in numerous vertebrate cells including neurons, muscle, endocrine, and hematopoietic. Each tissue in turn expresses a complex yet characteristic pattern of AChE forms which differ in oligomeric structure, hydrophobicity, and subcellular localization. We have studied the biogenesis of these multiple AChE forms to determine the molecular basis for this diversity and to understand how electrically excitable cells regulate and target identified synaptic components to highly specialized functional domains on their plasma membranes. We now present evidence that all AChE forms in electrically excitable cells are encoded by a single gene and therefore the diversity of molecular forms most likely arises by post-transcriptional and/or post-translational events including assembly and post-translational modifications.

Chicken and quail express two allelic forms of the AChE catalytic subunit polypeptide which differ by an apparent 5-10K daltons on SDS polyacrylamide gels. Individual quails express either the alpha (110 KDa) or beta (100 KDa) AChE subunits, or both. Our studies in quails show that the alpha and beta alleles occur with a frequency of 0.7 and 0.3 respectively in our sample population and in mating experiments segregate as co-dominant autosomal loci in classic Mendelian-fashion. Within individuals, neurons and muscle cells always express the same AChE alleles. Furthermore, the same alleles are expressed in all oligomeric AChE forms, whether globular or asymmetric. These studies indicate that all AChE forms in nerves and muscle are encoded by a single gene.

Newly synthesized AChE polypeptide chains in neurons and muscle appear identical by SDS gel electrophoresis. Unlike the mature AChE in chicken neurons, or the membrane-bound human erythrocyte AChE, the newly synthesized neuronal AChE molecules do not aggregate in the absence of detergents nor exhibit altered mobility by charge-shift gel electrophoresis indicating that they are not initially hydrophobic. It appears that the hydrophobic property of a subset of the neuronal AChE polypeptides is acquired during a subsequent maturational step. Muscle cells, on the other hand, do not appear to synthesize and assemble detectable quantities of the hydrophobic AChE polypeptide chains. The biogenesis of the various AChE forms will be discussed in relation to targeting these molecules to specialized regions of the plasma membrane in electrically excitable cells.

AVIAN ACETYLCHOLINESTERASE: MOLECULAR STUDIES ON THE STRUCTURE AND REGULATION OF THE ASYMMETRIC FORM IN MUSCLE.

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Acetylcholinesterase (AChE) in cholinergic tissues occurs as a
series of oligomeric forms. The largest of these forms contains a
dimensionally asymmetric (collagen-like) subunit in addition to the
globular catalytic subunits. These asymmetric forms are the
predominant species at the neuromuscular junction where their
localization and appearance are directly dependent upon functional
innervation. In birds or mammals these forms disappear following
denervation or blockage of neurotransmission. During embryonic
development, these forms become specifically localized at high density
on the synaptic basal lamina at the earliest stages of skeletal muscle
innervation.

To study the mechanisms underlying regulation of appearance and
localization of the asymmetric AChE forms we have purified this enzyme
from newly hatched chicken pectoral muscle using immunoaffinity
methods. The purified asymmetric AChE exhibits inhibition properties
of both AChE and butyrylcholinesterase (ChE) and contains three
subunits with apparent molecular weights of 110 kDa, 72 kDa and 58
kDa. Inhibitor binding and specificity to mAbs directed against AChE
and ChE indicate that the 110-kDa subunit contains the AChE catalytic
site whereas the 72-kDa subunit is a ChE catalytic subunit. All these
types of subunits (of this hybrid form) are linked together by
disulfide bonds. A model for this new "hybrid" asymmetric form found
in developing chicken muscle will be presented.

We have examined the expression of the AChE catalytic subunit
mRNA transcripts during muscle development by Northern hybridization
using a cDNA encoding the AChE catalytic subunit from chicken muscle.
cDNAs were isolated from a gt 10 cDNA library derived from 13-day
embryonic chicken muscle poly A+ RNA by cross hybridization using an
oligonucleotide probe synthesized from selected nucleic acid sequences
of the analogous enzyme from Torpedo californica. The isolated cDNAs
showed high regional homology with the deduced sequence from Torpedo
AChE, showed divergence in several of these homologous regions from
the deduced sequence for human brain ChE, and showed specificity to
transcripts for AChE but not ChE by Northern blot analysis. This cDNA
hybridizes with the highest intensity to three transcripts within 14-
day-embryonic poly A+ RNA in Northern blots from muscles of 10-day-
embryonic to newly-hatched chickens. Further analyses and
quantitation of the transcripts encoding these AChE subunits during
muscle development and synaptogenesis will be presented.

STRUCTURAL AND IMMUNOCHEMICAL PROPERTIES OF FETAL BOVINE SERUM ACETYLCHOLINESTERASE

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We have recently shown that there are structural and conformatic ial differences in the vicinity of the active center domains of fetal bovine serum acetylcholinesterase (FBS-AChE) and Torpedo AChE (Kopec et al. (1987) Fed. Proc. 46, 285). This conclusion was reached on the basis of determination of the sequence of the first 105 amino acid residues at the amino terminus of FBS-AChE and of the differential inhibition of catalytic activity of these two enzymes by the monoclonal antibody AE-2.   epitope for MAb AE-2 is located between amino acids 52 and 82 of FBS-AChE.

We have elucidated approximately 90% of the amino acid sequence of FBS-AChE and compared it with those of Torpedo AChE (Schumacher et al. (1986) Nature 319, 407) and human serum BuChE (Lockridge et al. (1987) JBC 262, 549). FBS AChE shows approximately 60% and 50% amino acid sequence homology with Torpedo AChE and with human serum BuChE, respectively. There are long stretches in the amino acid sequences of FBS-AChE and of Torpedo AChE in which very high sequence homology is observed, whereas other regions display little or no sequence homology. There are seven cysteine residues in FBS-AChE as against eight in Torpedo, Cys 231 being replaced by glycine. We have so far located five of the remaining six cysteine residues in FBS-AChE, all of which are located at the same positions as in the Torpedo enzyme, as is the active site serine. Comparison of the amino acid sequences of the three cholinesterases permits the proposal of candidate aspartic acid and histidine residues which may be involved in the charge relay systems of these serine esterases.

BIOGENESIS OF HUMAN CHOLINESTERASES: FROM GENE TO PROTEIN

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Cholinesterases (ChEs) are highly polymorphic serine hydrolases involved in the termination of neurotransmission in cholinergic synapses and neuromuscular junctions [1]. Both the levels and the molecular forms of ChEs were shown to be modulated during development, denervation and regeneration processes in various species [2]. In order to examine the molecular mechanisms underlying these phenomena in humans, we have used oligodeoxynucleotide probes to isolate full-length cDNA clones coding for human ChE [3,4]. These clones are currently employed in our laboratory to study the biogenesis of human ChE at various levels of gene expression.

Several genes encode for human cholinesterases. In situ hybridization of [^{35}S]-labled ChEcDNA to spread human chromosomes revealed sites for ChE genes on two different chromosomes, No. 3 and 16 [5,6]. Screening of several genomic DNA libraries, using [^{32}P]-ChEcDNA as a probe, enabled the isolation of genomic DNA fragments derived from at least 3 different genes, one of which (on chromosome 3)appears to contain multiple introns, whereas 2 others (on chromosomes 3 and 16) appear to be processed genes, originated by post-transcriptional events from the latter [7,8]. When combined with published genetic linkage evidence [9], these findings imply that at least 2 genes (the original one and a processed one) may direct the synthesis of active ChE in humans.

Active transcritpion of ChE genes in human oocytes. Oocytes from different species display cholinoceptive properties [10] and ChE activities [11]. Using in situ hybridiztion to frozen ovarian sections, we detected high amounts of ChEmRNA transcripts in developing human oocytes [12,13]. The enhanced transcription of ChE genes in oocytes suggests that ChEs are involved in oogenesis and/or sperm-egg interaction processes. It also implies that if processed ChEDNA sequences are re-inserted into the genome by post-transcriptional mechanisms, they would be inheritable, which may explain the origin of the processed ChE genes in humans.

Alternate transcription of ChE genes in nervous system tumors. Previous studies have shown distinct patterns of ChE molecular forms in various brain tumors [14] as compared with normal brain [15]. To examine whether these arise from alternative transcription of the ChE genes, cDNA libraries from various tissue origins were screened using labeled ChEcDNA and synthetic oligonucleotides. Evidence for both alternativa splicing and multiple termination sites was found in ChEcDNA clones from glioblastoma and neuroblastoma tumors, but not in normal tissues [16].

Synthetic ChEmRNA is sufficient to produce active ChE in microinjected oocytes, while additional mRNAs contribute to the tissue-specific properties of ChEs. Synthetic ChEmRNA, transcribed off the human ChEcDNA clone, was microinjected into Xenopus oocytes, where tissue ChEmRNA was shown to be translated into active ChE [17,18]. The clone-produced ChEmRNA was highly efficient in inducing the synthesis, assembly into dimers and membrane association of active ChE molecules with high affinity towards specific substrates and inhibitors. Co-injection with brain poly(A) RNA further enabled the assembly of ChE into tetramers, whereas with muscle poly(A) RNA heavier membrane-associated forms appeared [19]. Thus the tissue-specific expression patterns of ChE genes depend first on the mRNA coding for the catalytic ChE subunit but also on other mRNAs, probably coding for non-catalytic "tail" subunits and/or for proteins performing post-translational processes.

Antibodies to clone-produced human ChE interact with multiple ChE forms and with thyroglobulin. Bacterial expression of ChEcDNA enabled the production of ChE peptides and the elicitation of antibodies against those. These antibodies interact with various denatured and native forms of ChEs as well as with purified human thyroglobulin (Tg), which bears sequence homology with ChE [7,20]. Reciprocal experiments have shown that autoantibodies to Tg in hyperthyroid patients interact with the blotted ChE peptides as well as with ChE in endplates of fetal muscle fibers, which may explain the cholinergic-related symptoms implicated with hyperthyroidism (21).

Current research in our laboratory continues along these lines, aiming to relate the expression of particular ChE genes and their mRNA transcripts to defined tissue-specific molecular forms of ChE in humans.

References

1. Silver (1974) The Biology of Cholinesterases. North-Holland Publishers.
2. Massoulie, J. and Bon, S. (1982) Ann. Rev. Neurosci. 5, 57-106.
.3. Prody, C., Gnatt, A., Zevin-Sonkin, D., Koch, R., Goldberg, O. and Soreq, H. (1986) J. Neurosci. Res. 16, 25-36.
4. Prody, C., Zevin-Sonkin, D., Gnatt, A. Goldberg, O. and Soreq, H. (1987), PNAS, 84, 3555-3559.
5. Soreq, H., Zamir, R., Zevin-Sonkin, D. and Zakut, H. (1987) Human Genetics, in press.
6. Zamir et al., this conference.
7. Soreq, H. and Gnatt, A. (1987) Molecular Neurobiology, in press.
8. Gnatt and Soreq, this conference.
9. Lovrien, E. W., Magenis, R. E., Rivas, M. L., Lamvik, N., Rowe, S., Wood, J. and Hemmerling, S. (1978) Cytogenet. Cell Genet. 22, 324-326.
10. Kusano, K., Miledi, R. and Stinnakre, J. (1977) Nature 270, 739-741.
11. Soreq, H., Parvari, R. and Silman, I. (1982).
12. Malinger, G., Zakut, H. and Soreq, H., submitted.
13. Zakut et al., this conference.
14. Razon, N., Soreq, H., Roth, E., Bartal, A. and Silman, I. (1984) Exp. Neurol. 84, 681-695.
15. Zakut, H., Matzkel, A., Schejter, E., Avni, A. and Soreq, H. (1985) J. Neurochem. 45, 382-389.
16. Prody et al., this conference.
17. Soreq, H., Zevin-Sonkin, D. and Razon, N. (1984) EMBO J. 3, 1371-1375.
18. Soreq, H. (1985) CRC Crit. Rev. Biochem. 18, 199-238.
19. Dreyfus et al., this conference.
20. Schumacher, M., Camp, S., Maulet, Y., Newton, M., McPhee-Quigley, K., Taylor, S. S., Friedman, T. and Taylor, P. (1986) Nature 319, 407-409.
21. Zisling et al., this conference.

STRUCTURE-FUNCTION RELATIONSHIPS IN HUMAN CHOLINESTERASE APPROACHED BY MICROINJECTING
XENOPUS OOCYTES WITH CLONE-PRODUCED SP₆mRNA

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Cholinesterases (ChE) are ubiquitous, highly polymorphic serine hydrolases degrading
acetycholine. The various forms of ChE in different tissues may be distinguished by their
substrate specifities and sensitivity to selective inhibitors, as well as by their
subcellular and cell type localization and by the different nature and number of catalytic
and non-catalytic ("tail") subunits composing each form [1]. For example, the major ChE
form in the human serum is globular soluble tetramers of butyrylcholinesterase (BuChE),
highly sensitive to the organophosphorous compound iso-OMPA [2]. In contrast,
membrane-associated dimers of acetylcholinesterase (AChE) appear on red blood cells and
are sensitive to the quarternary compound BW284C51 [3]. Heavy "tailed" forms of
membrane-associated AChE are characteristic of muscle [4], whereas AChE tetramers are the
major ChE form in the human brain [5]. In order to approach the molecular mechanisms
underlying the biogenesis of the polymorphic human ChEs, a full length cDNA clone coding
for human serum BuChE [6] was subcloned into the SP₆ transcription vector [7]. Synthetic
polyadenylated ChEmRNA was transcribed in vitro off these constructs and microinjected
into Xenopus oocytes, where the translation of ChEmRNA [8], as well as the
post-translational processing and subcellular compartmentalization of the catalytically
active enzyme product take place [9,10].

When injected alone, the synthetic clone-produced ChEmRNA induced in the oocytes the
synthesis of ChE capable of degrading acetylthiocholine (AcThCh) and butyrylthiocholine
(BuThCh) in the range of micromoles/hr/ng of injecected SP₆ ChEmRNA, several orders of
magnitude higher than the activities produced in oocytes injected with non-enriched
poly(A) RNA [8-10]. The nascent cytoplasmic enzyme displayed km values of 6.3×10^{-4} M^{-1}
and 1.7×10^{-5} M^{-1} towards BuThCh and AcThCh, respectively, as expected from human BuChE
[11]. Also, the BuChE-specific organophosphorous inhibitor iso-OMPA (but not the
AChE-specific inhibitor BW284C51) blocked this ChE activity in the micromolar range,
similar to their action on serum BuC E [12]. In contrast, the membrane-associated fraction
of the enzyme displayed km values of ca. 2×10^{-5} M^{-1} towards both substrates, and was
sensitive to the AChE-specific inhibitor BW284C51 as well, at the same concentration
(1.10^{-5} M) as the red blood cell AChE.

Subcellular fractionation of the SP₆ ChEmRNA injected oocytes, followed by sucrose
gradient centrifugation and activity measurements, revealed that the clone-produced enzyme
appears as assembled dimers in the oocyte's cytoplasm, membrane-associated fraction and
incubation medium. Co-injection of SP₆ ChEmRNA with unfractionated total poly(A)+ brain
RNA induced the formation of ChE tetramers, sedimenting as 12 S, in the
membrane-associated fraction. When liver poly(A)+ RNA was co-injected, the activity was
mainly constituted of light forms (mostly monomers and dimers). Finally, co-injection with
muscle poly(A)+RNA resulted in the appearance of heavy, perhaps tailed forms, associated
with the membrane of the injected oocytes. Altogether, these findings demonstrate that:

a) SP_6 ChEmRNA by itself is sufficient for the biosynthesis, assembly into dimers and association with the membrane of active ChE;

b) The affinity of BuChE towards specific substrates and its sensitivity to organophosphates are most probably inherent properties of the primary amino acid sequence, whereas the sensitivity to the reversible AChE inhibitor BW284C51 may be acquired through post-translational processing; and

c) The correct formation of multi-subunit molecular forms of ChE in particular tissues requires both ChEmRNA and other, tissue-specific proteins, that are absent from Xenopus oocytes.

References

1. Massoulie, J. and Bon, S. (1982) Ann. Rev. Neurosci. 5, 57-106.
2. Lockridge, O. and LaDu, B. N. (1986) Biochem. Genetics 24, 485-498.
3. Dutta-Choudhury, T. A. and Rosenberry, T. (1984) J. Biol. Chem. 259, 5653-5660.
4. Dreyfus, P. A., Rieger, F. and Pincon-Raymond, M. (1983) PNAS 80, 6698-6702.
5. Zakut, H., Matzkel, A., Schejter, E., Avni, A. and Soreq, H. (1985) J. Neurochem. 45. 382-389.
6. Prody, C., Zevin-Sonkin, D., Gnatt, A., Goldberg, O. and Soreq, H. (1987) PNAS 84, 3555-3559.
7. Krieg, P. A. and Melton, D. A. (1984) Nuc. Ac. Res. 12, 7057-7070.
8. Soreq, H., Parvari, R. and Silman, I. (1982) PNAS 79, 830-835.
9. Soreq, H., Zevin-Sonkin, D. and Razon, N. (1984) The EMBO J. 3, 1371-1375.
10. Soreq, H. (1985) CRC Crit. Rev. Biochem. 18, 199-238.
11. Aldridge, W. N. and Reiner, E. (1972) Enzyme Inhibitors as substrates. North-Holland Publishing Co., Amsterdam.
12. Razon, N., Soreq, H., Roth, E., Bartal, A. and Silman, I. (1984) Exp. Neurol. 84, 681-695.

COVALENTLY ATTACHED PHOSPHATIDYLINOSITOL AS THE MEMBRANE-ANCHORING
DOMAIN OF TORPEDO ACETYLCHOLINESTERASE

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Acetylcholinesterase (AChE) is one of the growing group of proteins which are
attached to the plasma-membrane via covalently bound PI. Evidence for this
role of PI comes from two experimental approaches: 1) Selective release of
these proteins by a phosphatidylinositol-specific phospholipase C (PIPLC)
from Staphylococcus aureus; 2) Chemical analysis of their membrane-anchoring
domain. AChE from Torpedo electric organ was one of the first such proteins
to be described. Hydrophobic forms of Torpedo AChE can be selectively
released from membranes on addition of PIPLC; in addition, chemical analysis
of the membrane-anchoring domain of AChE reveals stoichiometric amounts of
inositol phosphate covalently attached, by a glycosidic linkage, to a non-
acetylated sugar residue, which is itself attached to the protein polypeptide
backbone, probably via an intervening oligoglycan. Further, approximately 3
moles of phosphate are found in this domain and GC/MS analysis reveals that
the PI consists of a mixture of fatty acids, of which stearate and palmitate
are major components. Evidence will be presented for homology between AChE
and a number of other proteins anchored via PI.

In order to examine the localization, distribution, sites of biosynthe-
sis and ontogeny of the post-translational addition of the PI-glycolipid to
the polypeptide backbone of the AChE catalytic subunit, the susceptibility of
AChE to PIPLC during the embryonic development of Torpedo electric organ and
in the electromotor system and other excitable tissues of the adult Torpedo
ocel ata was tested. PIPLC solubilizes significant amounts of the membrane-
bound form of AChE throughout embryonic development of the electric organ, as
it does in the adult electric organ. In the electromotor system of the
mature fish, PIPLC solubilizes almost quantitatively the AChE dimer in the
electromotor axon as in the electric organ itself, but the corresponding
fraction in the electric lobe is almost totally resistant to the phospholi-
pase. This finding implies that the covalently bound phosphatidylinositol is
added concomitantly with axonal transport. A substantial part of the mem-
brane-bound AChE in back muscle is sensitive to PIPLC, whereas the membrane-
bound form in Torpedo brain is completely resistant.

The possible functional significance of using PI as a membrane anchor
for AChE will be discussed.

THE VOLTAGE-DEPENDENCE OF TRANSMITTER RELEASE

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In 1981, Llinás et al. (Biophys. J. 33:323) reported that transmitter release at the squid giant synapse appeared to be voltage-dependent. Large depolarizations approaching the calcium equilibrium potential admit the same calcium influx presynaptically during the pulse as small ones, but the larger pulses released more transmitter. Subsequently, Augustine et al. (J. Physiol., Lond. 367:163, 1985) showed that this was partly due to the large calcium tail current following large pulses, and reduced the effect by restricting attention only to release during the pulse. They further found that by considering synaptic delay and plotting release at a short time after presynaptic current, this voltage-dependence sometimes vanished.

Meanwhile, Simon and Llinás (Biophys. J. 48:485, 1985) and Fogelson and I (Biophys. J. 48:1003, 1985) developed ways of predicting the local presynaptic calcium concentration at release sites near calcium channels. We considered the diffusion of calcium after influx through channels into cytoplasm, with binding to cytoplasmic buffer. Calcium was removed by uptake into organelles, or extrusion by surface pumps. All parameters had been measured independently. Calcium channels and release sites were located according to microscopic evidence.

Simulations using these models showed that large depolarizations lead to different distributions of presynaptic calcium than do small ones. Large depolarizations open more channels, with less influx per channel, leading to a more uniform distribution of submembrane calcium. Simon and Llinás considered that the power-law dependence of transmitter release might indicate activation of vesicles at multiple points, leading to more effective release when many channels open near a vesicle. I and Fogelson (Proc. Natl. Acad. Sci. USA 83:3032, 1986) considered that the nonlinear dependence of release on external calcium concentration might reflect cooperativity of calcium action at single sites, and that higher voltages opening more channels cause the calcium domains, or clouds of calcium ions surrounding channel mouths, to overlap and result in higher calcium concentrations at release sites. Both models predict a larger transmitter release at higher voltages admitting the same overall amount of calcium presynaptically as lower voltages, without implicating any voltage-sensitive step in transmitter release.

Parnas and Parnas have performed a very different series of experiments on neuromuscular junctions (Parnas and Parnas, J. Physiol., Paris 81:289, 1986), suggesting that in addition to the effect of calcium on release, voltage directly controls the release process, too. They propose that presynaptic calcium enables release, but that in addition presynaptic depolarization is required for exocytosis. We tested this hypothesis (Zucker and Landò, Science 231:574, 1986) by using hyperosmotic treatment or mitochondrial uncouplers to elevate presynaptic calcium sufficiently to trigger substantial spontaneous release. Calcium influx was prevented by chelating external calcium. Depolarization then leads to calcium efflux through open calcium channels, and this effect can be blocked with calcium antagonists (Landò et al., J. Neurobiol. 17:707, 1986). However, depolarization failed to evoke release, even when presynaptic calcium was high. Controls showed that the treatments used did not block the phasic

release process.

We re-evaluated the evidence that had been presented in support of a direct effect of voltage. Macro-patch electrodes were used to excite nerve terminals twice. As the first pulse increased, release increased while facilitation measured by the second pulse decreased. Although this could indicate an increase in release while calcium influx decreased in the first pulse, we found evidence that pulses of different amplitudes preferentially activate different terminals. Increasing the first pulse recruited distant terminals, which were not tested by a small second pulse. A large second pulse testing these terminals showed facilitation to increase as the first pulse increased. When the first pulse is given intracellularly and the test pulse still uses a macro-patch electrode, the same interpretation of the results is possible (Zucker et al., J. Physiol., Paris 81:237, 1986).

Another experiment indicated that a small pulse alone released almost no transmitter, while after a spike train it triggered massive release. We found this result to reflect changes in terminal excitability following the train, such that a subthreshold pulse triggered an action potential after a train. Similar, but less dramatic results, could be obtained in tetrodotoxin, where calcium spikes at nerve terminals can still occur.

The time course of transmitter release remains remarkably invariant with changes of calcium influx and resting calcium. Although some formulations of the calcium hypothesis of release contradict this, our simulations of presynaptic calcium transients indicate that this result is expected, especially if the rise in active calcium is not the rate-limiting step in exocytosis.

Small hyperpolarizing pulses just before or after a depolarizing pulse can have large effects on release by the pulse, but little effect on subsequent release by a second test pulse. This was taken to indicate that the small hyperpolarizations affected release directly without affecting calcium influx and subsequent facilitation. However, it is easily shown (Zucker, Biophys. J., in press, 1987) that small changes in calcium influx will have large effects on release but very small and probably undetectable effects on subsequent facilitation.

In conclusion, there is no evidence that presynaptic potential during a spike has any effect on transmitter release beyond its effect on calcium entry.

Short Term and long term regulation of transmitter release. By
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The synapse is the site of many short term and long term changes in
transmission efficiency. In this communication we concentrate on four recent
findings regarding the presynaptic nerve terminal and transmitter liberation.
1. Extracellular calcium inhomogeneity. Clamping the extracellular free
calcium concentration ($[Ca]_o$) to values found in the bulk solution by calcium
buffers, produces a reduction in quantal transmitter liberation. This
indicates that $[Ca]_o$ is higher near the release sites (presumably the synaptic
cleft), than in the bulk extracellular medium. We do not know whether this
inhomogeneity is due to localized extrusion processes directed towards the
synaptic cleft generating a standing concentration gradient or due to local
calcium binding molecules. Whatever the cause for this inhomogeneity will
turn out to be, it can produce long term changes in the amount of transmitter
liberated and thus can regulate the synaptic efficiency.
2. Slow depression in transmitter release. Prolonged stimulation of the
presynaptic nerve terminal at low rates (0.067 to 0.5 Hz) produces a slow
decrease in the end plate potential amplitude at the frog neuromuscular
junction blocked by d-tubocurarine or α-bungarotoxin. This slow depression
which can reach 50% of the initial response, develops over many minutes and is
of presynaptic origin. Fluctuation analysis by the group regression method
shows that it is due to a reduction in the number of quanta released by the
nerve impulse. Furthermore, ionophoretic application of acetylcholine does
not reveal any substantial change in the post synaptic sensitivity. This slow
depression seems to be an additional frequency modulator of synaptic
transmission.

3. The phosphoinositide pathway. In recent years there is a great renewed interest in the phosphoinositide second messenger system, which employs two intracellular signals: inositol 1,4,5 triphosphate (IP_3) and 1,2 diacylglycerol (DG). The action of DG in the activation of a calcium-phospholipid dependent protein kinase C (PKC) can be mimicked by the phorbol ester TPA (12-0-tetradecanoyl phorbol-13 acetate). We show that application of TPA causes an increase in the end plate potential amplitude at the frog neuromuscular junction. This increase in amplitude is due to an increase in the number of acetylcholine quanta liberated by the nerve impulse, without any appreciable change in post synaptic sensitivity. Spontaneous transmitter release is also augmented in parallel with evoked release. Synaptic depression is deepened by TPA. These results indicate that the DG branch of the phosphoinositide system can be used in regulation of synaptic transmission; its natural activator is not yet known.

4. Channels in synaptic vesicles. Synaptic vesicles are considered to be the subcellular structures responsible for quantal transmitter liberation; but their small size and intracellular localization made them difficult for a direct electrophysiological study. We used a fused vesicle preparation to obtain large structures suitable for patch recording. Three types of electrical activity were observed, indicating that the vesicle membrane possesses conductances to K^+, Na^+ and Cl^- ions. We envisage that the observed channels may participate in vesicle function and thus in the regulation of transmitter liberation.

CALCIUM BUFFERING AND CALCIUM MEASUREMENTS IN SINGLE SECRETORY
CELLS UNDER PATCH CLAMP.

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Various ways of stimulating mast cells lead to a stereotyped
calcium signal (1,2) as measured by the fluorescent indicator dye
fura-2 (3). A prominent transient increase in free intracellular
calcium concentration, lasting two to five seconds, is followed
by a prolongued phase of elevated calcium. The transient is most
probably due to calcium released from intracellular stores
because i) it can also be elicited by intracellularly admi-
nistered Inositoltrisphosphate (IP_3), ii) it is independent
of extracellular calcium, and iii) no ionic currents can be
observed which could mediate calcium entry. If the rising phase
of the transient were due to electrogenic calcium entry through
the plasma membrane, currents in the order of magnitude 10-100 pA
would be expected in a given cell. Intracellularly administered
Ca/EGTA buffers have to be in the concentration range 1 to 10 mM
in order to reliably suppress the calcium transients (4). The
transients also allow an order of magnitude estimate of the
cell's high-affinity calcium buffer concentration, which turns
out to be in the range 200 μM to 1 mM.

The origin of the prolongued phase of calcium elevation is not
clear. It is found, however, that $[Ca]_i$ following a transient can
be quite markedly influenced by membrane voltage (4), which
points towards an involvement of ionic channels or other membrane
transport mechanisms during that phase. Estimates of membrane
currents which would be required to maintain such elevated
calcium levels (if they were due to calcium entering through
channels) are in the range 1 pA per cell. Whole-cell currents of
such small amplitude are hard to detect above the background of
unspecific conductances.

(1) Neher, E. & Almers, W. (1986). EMBO J. 5, 51-53.

(2) Penner, R., Pusch, M. & Neher, E. (1987). Bioscience Reports,
    (in press).

(3) Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985). J. Biol.
    Chem. 260, 3440-3450.

(4) Neher, E. (1987). J. Physiol. (in press).

NEUROMUSCULAR TRANSMISSION IN MUSCLES PARALYSED BY BUTANE DIONE MONOXIME

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The chemical phosphatase, butane dione monoxime (BDM) depresses
surface membrane calcium currents and contraction in cardiac muscle
and the two effects are thought to be related (Wiggins et al., 1980).
We have found that BDM also depresses contraction in mammalian
skeletal muscle, whether elicited by indirect stimulation, direct
stimulation or by raising the extracellular potassium concentration:
twitches, tetanic contractions and potassium contractures are all
depressed and the effects are reversible. As the resting membrane
potential and action potentials are essentially normal in the
presence of BDM, it was concluded that BDM depresses contraction by
affecting intracellular steps in excitation-contraction coupling.
Neuromuscular transmission persisted in muscles paralysed by BDM (2-
20 mM). There was little change in the time course, amplitude or
frequency of spontaneous miniature endplate currents or potentials
but the amplitude of endplate currents and potentials increased,
indicating an increase in acetylcholine secretion. It is intriguing
that a drug that blocks calcium currents should increase transmitter
secretion: presumably, it does not block calcium currents in nerve
terminals. BDM should be an invaluable tool for paralysing muscles
in investigations of neuromuscular transmission.

Wiggins, J.R., Raiser, J., Fitzpatrick, D.F. and Bergay, J.L. (1980)
 Inotropic actions of diacetyl monoxime in cat ventricular
 muscle. *J. Pharmacol and Exp. Ther.* 212: 217-224.

COMPLEXITY AND REGULATION IN THE ACETYLCHOLINE STORAGE SYSTEM OF SYNAPTIC VESICLES

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The acetylcholine(ACh) storage system of Torpedo electric organ synaptic vesicles is composed of an ATPase, an ACh transporter and a receptor for the inhibitory compound 2-(4-phenylpiperidino)cyclohexanol (vesamicol; formerly AH5183). The vesamicol receptor is a very stable protein containing a critical SH group and a carboxyl group of pKa 6.26 ± 0.03 which probably ion pairs in a hydrophobic environment with the bound drug. Chemical modification, proteolysis and pH titration approaches were used to demonstrate this. The drug binding site was shown to be on the cytoplasmic surface of the vesicle membrane by use of a membrane-impermeant analogue of vesamicol. Rate processes for association and dissociation of vesamicol were studied and found to be heterogeneous in some cases, suggesting that multiple forms of the receptor can exist.

When crude vesicles were banded in an isosmotic sucrose density gradient both classical fully loaded VP_1 and recycling VP_2 vesicles could be observed to actively transport $[^3H]ACh$. The properties of the vesamicol receptor (measured with $[^3H]$vesamicol binding) and the amount of the SV2 antigen characteristic of many neurosecretory vesicles were studied in both vesicle types. Relative to VP_1 vesicles, the VP_2 vesicles had a ratio of ACh transport activity to apparent vesamicol receptor concentration which typically was 4 to 7-fold higher, whereas the ratio of SV2 antigen concentration to apparent vesamicol receptor concentration was about 2-fold higher. The Hill coefficient and equilibrium dissociation constants for vesamicol binding to VP_1 and VP_2 vesicles were essentially the same at 2.0 and 19 nM, respectively. This study demonstrated that the vesamicol receptor ACh storage system appears to be regulated in a manner which survives homogenization of the tissue.

A transmembrane signal has been found to regulate the vesamicol receptor. Typical intact VP_1 vesicle preparations express about 200 pmol receptor/mg protein, whereas hyposmotically lysed, resealed vesicle ghosts express up to 600 pmol/mg or 12 receptors/vesicle. $[^3H]$vesamicol binds to intact VP_1 vesicles in a manner essentially unaffected by exogenous ACh, but binding to lysed vesicles is blocked by 5 to 50 mM ACh in a positively cooperative noncompetitive manner. Previously bound $[^3H]$vesamicol is dissociated by added ACh with a several minute lag period, which suggests that a significant reorganization in the ACh binding site occurs before it can cause vesamicol dissociation. Since vesicle ghosts are sealed (demonstrated with $[^{14}C]$ribitol) this might appear to suggest that the linked ACh site is on the outside of the membrane. However, in ghosts the vesicle membrane was rapidly (<3 sec) and fully permeable to as much as 0.3 M exogenous ACh. The results demonstrate that much of the vesamicol receptor in intact VP_1 vesicles often is cryptic because of the presence of a factor inside of the vesicles which can be released by hyposmotic lysis and that the factor might be internal ACh.

Finally, it has been demonstrated that the vesamicol receptor is linked indirectly to ACh transport inhibition. Binding of [^{3}H]vesamicol and inhibition of [^{14}C]ACh transport are coincident at high VP_1 vesicle concentration, but at low vesicle concentration, ACh transport inhibition occurs when only a small fraction of the receptor sites have been occupied. This behavior is apparently time independent. Also, at very low vesicle concentration some very high affinity (K_d less than 1nM) vesamicol receptor clearly is expressed. This suggests that a dissociable factor which weakens the vesimacol receptor affinity copurifies with the vesicles, or that the vesicles interact with each other through the vesamicol receptor. Using trace [^{3}H]vesamicol (not enough to inhibit ACh transport) and [^{14}C]ACh, it was shown that a biotinylated vesamicol analogue occupied the receptor and inhibited [^{14}C]ACh transport. Addition of excess avidin "neutralized" the biotinylated vesamicol and freed the receptor, yet [^{14}C]ACh transport did not reactivate for at least the next 50 min. It does reactivate after much longer time periods or in vivo. Thus, although drug binds with equilibrium behavior, the transport inhibition is irreversible over the relevant time scale. Using trace levels of [^{3}H]vesamicol, [^{14}C]ACh and nonlabeled drug analogues as competitors (varied from zero to high concentrations), it was shown that at low vesicle concentration the shift in the midpoint of the [^{3}H]vesamicol dislacment curve from the [^{14}C]ACh transport inhibition curve depends on the analogue studied. For example, it is 3-fold for l-vesamicol (the active enantiomer), 6-fold for d-vesamicol and 10-fold for deoxyvesamicol. This relationship held for 16 vesamicol analogues and 9 other compounds of widely varying structures. Only for a weakly binding dimer of vesamicol, hexamethonium and chloroquine did the two curves fall in reverse order, and in no case was there a potent compound able to block [^{14}C]ACh uptake without displacing [^{3}H]vesamicol within a factor of 10-fold higher concentration of the ligand. Thus, there is no evidence that another ligand binding site critical to ACh storage exists. The result also suggest that an induced conformational change in the receptor dependent on the drug structure links the vesamicol receptor and ACh transport inhibition. Lastly, in lysed vesicles where ACh inhibits [^{3}H]vesamicol binding, the inhibition was shown to not occur at very low vesicle concentration. This suggests that an external dissociable factor which copurified with the vesicles mediates linkage between the ACh and vesamicol binding sites.

In summary, these results strongly suggest that the ACh vesicular storage system is physiologically regulated and complex. At least one osmotically active internal factor which might be ACh controls the conformation of the vesamicol receptor on the outside of the vesicle. An external dissociable factor mediates this signal and the affinity of the receptor for vesamicol. A possible physiological role for such a signaling device will be discussed.

THE MECHANISM OF ACETYLCHOLINE RELEASE : ITS ESSENTIAL COMPONENT THE MEDIATOPHORE

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Torpedo electric organ synaptosomes have proven to be very convenient for studying the mechanism of acetylcholine release. Ultrastructural changes taking place in the nerve terminal membrane while it releases transmitter suggest that a category of large intramembrane particles which become more numerous support the translocation of acetylcholine upon calcium action. The involvement of integral membrane proteins in the release process was also demonstrated by incorporating them into artificial membranes which inherited from the native membrane the property of releasing acetylcholine in response to a calcium influx. This observation led us to purify from synaptosomal membranes the protein called mediatophore which is involved in the release process. A doughnut shaped molecule of about 200 000 d made of subunits at 15-17 000 d could be characterized. It has several essential physiological and pharmacological properties attributed to the mechanism of acetylcholine release.

MOLECULAR PROPERTIES OF THE SODIUM-CALCIUM EXCHANGER.

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The Na^+-Ca^{2+} exchanger is one of the major Ca^{2+} transporting molecules in excitable cells. To date, no inhibitor, toxin or affinity probe is known that could label the protein moiety associated with the Na^+-Ca^{2+} antiport activity and identify it. Therefore enriched appearance of a protein band on SDS-polyacrylamide gels in conjunction with increased specific transport activity served as a sole criterion for its purification and identification. Recently we have identified the protein moiety responsible for Na^+-Ca^{2+} exchange activity in synaptic plasma membranes (SPM). This was done by raising polyclonal antibodies in rabbits against each one of the detectable proteins present in the purified preparation containing the enriched specific transport activity. Two of the antibody preparations bound specifically to native SPM: antibodies which were raised against the 70,000 Da protein (the most prominent species consistently present in the purified preparation), and antibodies raised against a 33,000 Da protein (inconsistently present in variable amounts in the purified preparation). Both antibodies bound exclusively to a protein of 70,000 Da in native SPM. When, however, the purified 33,000 and 70,000 Da proteins were used as antigens, each one of the antibody preparations bound to both proteins.

In addition, both antibody preparations immunoprecipitated Na^+ gradient-dependent Ca^{2+} transport activity from solubilized SPM. This was obtained by incubation of solubilized SPM with a complex containing antibodies bound to Protein A-Sepharose beads, reconstitution of the material excluded from the beads and determination of the residual transport activity. The decrease in Na^+ gradient-dependent Ca^{2+} transport activity paralleled the amount of antibody bound to Protein A-Sepharose beads and could reach 82% as compared to the activity remaining in control experiments using preimmune sera. In comparison, ATP-dependent Ca^{2+} transport activity was unimpaired.

These results indicate that the 70,000 Da protein in SPM contains the catalytic Na^+-Ca^{2+} antiport activity. The presence of the 33,000 Da protein in some preparations and its properties may be explained by its being either a degradation product or a subunit of the 70,000 Da protein.

The purified 70,000 Da protein is a glycoprotein as revealed by treatment of the purified 70,000 Da species with endoglycosidase F. The deglycosylated protein migrates on SDS-polyacrylamide gels as an approximately 50,000 Da protein. Limited proteolysis by V8 protease or trypsin reveals that the polyclonal antibody preparation binds also to some of the larger peptide fragments obtained.

The stoichiometry of the Na^+-Ca^{2+} exchanger from the synaptic plasma membranes was studied in both native and reconstituted preparations. In kinetic experiments performed with the native preparation, initial rates of Na^+ gradient-dependent Ca^{2+} influx were compared to Ca^{2+} dependent Na^+ efflux. These experiments showed that 4.82 Na^+ ions are exchanged for each Ca^{2+} ion. Thermodynamic approach in which equilibrium measurements were done with the reconstituted preparation resulted in similar (4.76) stoichiometry. The effects of membrane potential, employing valinomycin induced K^+ fluxes could be demonstrated in the reconstituted preparation. The stoichiometry of the Na^+-Ca^{2+} exchanger remained the same both under conditions of positive inside or negative inside polarization of the vesicles membrane. The direct contribution of the Na^+-Ca^{2+} exchanger to the membrane potential across the reconstituted vesicle membrane could be demonstrated by using the lipophylic cation tetraphenylphosphonium.

Positive inside membrane polarization did not change the K_m to Ca^{2+} when Na^+ gradient dependent Ca^{2+} influx was measured. It led however to a 4-fold increase in the V_{max} of the process. When the ratio of internal to external Na^+ concentration was varied, positive inside membrane polarization led to a reduction by a factor of approximately 20 in the extent of Na^+ gradient required for half apparent maximal reaction velocity to be reached.

POTASSIUM CHANNELS IN SYNAPTOSOMES: PHARMACOLOGY AND TOXICOLOGY.

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Tracer ^{42}K and ^{86}Rb efflux methods have been developed to study the properties of K channels in isolated rat brain presynaptic nerve terminals (synaptosomes). On the basis of blocking pharmacology and characteristic tracer kinetic profiles, we have identified six different types of K channels: 1) Resting K channels represented by the efflux into media containing 5 mM K. This conductance is relatively insensitive to K channel blockers. 2) A depolarization-gated, rapidly inactivating (1 sec) K conductance that corresponds to the A current. This is selectively blocked by 4-aminopyridine (< 1 mM) and by - and - DaTX, two polypeptide components of _Dendroaspis angusciceps_ venom (dendrotoxins). 3) A depolarization-gated, non-inactivating K conductance that corresponds to the delayed rectifier. This is selectively blocked by phencyclidine (PCP) and dexoxadrol, by ß- and -DaTX, and by venoms from several scorpions including _T. serrulatus, C. sculpturatus,_ and _L. quinquestriatus._ 4 and 5) Two Ca-activated K channels that are selectively blocked by low concentrations of quinine (1 uM), and the neuroleptics, haloperidol and the phenothiazines. There are two components, with different sensitivities to the phenothiazines (IC_{50} = 20-30 nM and 1-5 uM). A component of _L. quinquestriatus_ venom blocks some of this conductance. 6) A K conductance that is selectively activated by certain sigma opioids and by levoxadrol (but not by its enantiomer, dexoxadrol). The ability of these agents to open this K channel can be blocked by naloxone.

The two voltage-gated K channels in hippocampus and corpus striatum nerve endings apparently can be modulated by protein kinase C, since diacyl glycerol causes a time-dependent inhibition of these channels. The data suggests that phosphorylation may be involved in regulating these channels.

We have begun to determine the chemical nature of some of these K channels by labelling them covalently with ^{3}H-azido PCP and with ^{125}I-labelled dendrotoxins.

PRESYNAPTIC CURRENTS IN FROG MOTOR NERVE TERMINALS

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Transmitter release from presynaptic nerve terminals is strongly influenced by the waveform of nerve terminal polarization following the arrival of an action potential. This, in turn, is largely determined by the properties of ionic conductances present in the nerve terminal membrane.

The small caliber of vertebrate motor nerve terminals precludes direct analysis of membrane currents by standard current- and voltage-clamp techniques. A more indirect approach is provided by the perineurial sheath surrounding motor axons (but not their terminals), which forms an electrically insulated "tube" around motor fibre bundles. Thus, an electrode positioned in this "tube" records extracellular currents flowing between axons and their terminals. Using this approach several groups have analyzed presynaptic spike currents in mammalian and amphibian motor nerve terminals. Their results suggest the existance of multiple K^+ (gK^+) and Ca^{++} (gCa^{++}) conductances in this structure, which may be modulated by neurotransmitters, neurotoxins, and nerve activity. Here we shall describe in some detail our analysis of presynaptic conductances in frog cutaneous pectoris motor nerve terminals.

Recordings from nerve bundles containing one or several motor axons disclose a "fast" gK^+ in the corresponding nerve terminals, which may contribute to rapid spike repolarization. This gK^+ persists in Ca^{++}-free solutions but is blocked by 10 uM or more of 3,4-diaminopyridine (DAP). Following DAP application, a second gK^+ is revealed, which has a slower time course, and is blocked by exposure to Mn^{++} or Cd^{++} and by extracellular Ca^{++} washout. This presumed Ca^{++}-activated gK^+ is suppressed also by 1-5 mM tetraethylammonium (TEA), which also block the "fast" gK^+. Both presynaptic gK^+s are sensitive to blockade by the neuromuscular transmitter acetylcholine (ACh). However, the high (0.5-2 mM) ACh doses required for this action and its insensitivity to d-tubocurarine and atropine, suggest it is not exerted via classical cholinergic receptors.

When preparations are incubated in 10 mM TEA, which presumably blocks most gK^+ in the motor nerve terminals, large signals reflecting inward currents at the terminals can be recorded. They are identified as presynaptic Ca^{++} currents by several criteria: a) their magnitude is positively related to $[Ca^{++}]_o$; b) they are reversibly reduced by Mn^{++} or Cd^{++}; c) they persist when Ca^{++} is replaced by Sr^{++}; d) they are reduced by verapamil (provided the nerve terminals are depolarized by elevated $[K^+]_o$). These presumed Ca^{++} currents are not blocked by the dihidropyridine antagonist nifedipine, suggesting the presynaptic Ca^{++} channel type is not the ubiquitous "L" type.

Repetitive (20-100 Hz) motor nerve activation in standard Ringer solution induces the buildup of presynaptic currents,

which resemble the presumed Ca^{++} currents encountered in TEA. They are similarly reduced by lowering $[Ca^{++}]_0$ or by adding Mn^{++} or Cd^{++}. It is possible that repetitive nerve activation causes partial gK inactivation, thereby prolonging presynaptic spike depolarization and Ca^{++} influx. Such a mechanism may contribute to frequency modulation of transmitter release from motor nerve terminals.

MOLECULAR STUDIES OF THE POSTSYNAPTIC 43K PROTEIN AND CALCIUM CHANNELS FROM SKELETAL MUSCLE.

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Proper alignment of macromolecules at the neuromuscular junction is necessary for rapid signal transmission across the synapse. For example, high concentrations of acetylcholine receptors occur on the crests of postjunctional folds directly across the synaptic gap from presynaptic active zones where voltage-sensitive calcium channels are thought to be localized. The cellular mechanisms responsible for forming and maintaining the specialized distributions of these two ion channels are largely unknown. We have examined peripheral membrane proteins of the postsynaptic membrane thought to be involved in anchoring receptors at synaptic sites and have also begun to develop immunological probes for voltage-activated calcium channels.

The Torpedo electrocyte postsynaptic membrane contains, in addition to the acetylcholine receptor, a major, peripheral membrane protein of M_r 43,000 (43K protein). Indirect evidence has implicated this protein in the mechanisms that restrict receptor mobility. In Torpedo electrocytes, the 43K protein is coextensively distributed with the receptor, can be chemically crosslinked to the beta subunit, and occurs in concentrations approximately equimolar with the receptor. This stoichiometric relationship is found throughout electric organ development of the electric organ (studies done in collaboration with V. Witzemann). The receptor and the 43K protein increase in a parallel manner from approximately 0.2-0.4 pmoles/mg protein in 44mm embryos to approximately 200 pmoles/mg in adult tissue. At each developmental stage examined, the equimolar ratio of 43K protein and AChR was maintained.

Monoclonal antibodies to the Torpedo 43K protein recognize a protein of similar size in mammalian muscle cells. The 43K protein is associated with patches of AChR that arise spontaneously on C2 myotubes. In these cells, the 43K protein and AChR are coordinately expressed in stoichiometric amounts. In collaboration with Zach Hall's laboratory, we have examined the expression of the 43K protein in genetic variants of C2 cells that lack functional AChR. In one variant that fails to synthesize the alpha subunit, 43K protein was expressed at approximately one-third the level found in wild-type cells. The variant did express the AChR beta subunit at levels comparable to that found for the 43K protein. These results suggest that expression of the receptor and the 43K protein are coordinated and may be subject to the same regulatory controls.

To examine the distribution and molecular structure of calcium channels, we have prepared monoclonal antibodies to the dihydropyridine (DHP)-binding complex/calcium channel from rabbit muscle transverse tubules. Although the t-tubule calcium channels are pharmacologically distinct from those found in most nerve terminals, structural similarities may exist. One monoclonal antibody, mab 1A, immunoprecipitates the digitonin-solubilized DHP-binding complex. This complex, when purified by WGA chromatography, sediments as a 21S component. The sedimentation coefficient is increased to about 24S after incubation with mab 1A IgG. Four polypeptides with apparent molecular weights under nonreducing conditions of 220kDa, 200kDa, 61kDa, and 33kDa cosediment with the 21S complex. Mab 1A recognizes the 200kDa polypeptide, as shown by Western blotting analysis. The electrophoretic mobility of this protein is unaffected by disulfide bond reduction. DHP-binding complex purified by WGA chromatography followed by immunoaffinity chromatography on a mab 1A column is comprised primarily of the same four polypeptides. Thus, the 200 kDa protein is a component of the DHP-binding complex from rabbit skeletal muscle, and in association with the other polypeptides, may comprise the voltage-sensitive calcium channel.

ACTION OF ANTICHOLINESTERASES ON ACETYLCHOLINE RECEPTORS

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The interactions of carbamate and organophosphate anticholinesterases with the nicotinic acetylcholine (ACh) receptor of *Torpedo* electric organ and muscarinic receptors of rat brain and neuroblastoma N1E-115 cell culture were studied. The effects on the nicotinic receptor were investigated by the changes occurring in its binding of ^{125}I-α-bungarotoxin and ^3H-phencyclidine and in receptor regulated $^{22}Na^+$ flux, and on the muscarinic receptor by changes in its binding of ^3H-quinuclidinyl benzilate and ^3H-cis-methyldioxolane and in ^3H-cGMP synthesis.

On the nicotinic receptor, the organophosphates soman and echothiophate interacted with the ACh binding site, acting as partial agonists, while VX was an allosteric inhibitor of the receptor's open channel conformation. The carbamates neostigmine and pyridostigmine acted as partial agonists while physostigmine was an open channel blocker. Tetraalkylammonium anticholinesterases interacted with the different receptor sites. Tetramethylammonium acted as an agonist. Increasing the chain length of the symmetrically substituted tetralkylammonium compounds increased the affinity of the receptor's allosteric site for them but decreased the affinity of the ACh-binding site. Edrophonium acted as a depolarizing blocker.

The population of muscarinic receptor with a very high affinity for ^3H-cis-methyldioxolane had a very high affinity for certain organophosphates (e.g. VX). The carbamates also inhibited the muscarinic receptors but only at very high concentrations.

Oxadiazolidinones, the nonesteratic irreversible inhibitors of ACh-esterase reacted reversibly, but with much l..er potency with both nicotinic and muscarinic ACh receptors.

The nicotinic ACh receptor of *Torpedo* electric organ, was used to develop a biosensor for organophosphate nerve agents. The pure ACh-receptor alone and with ACh-esterase were incorporated into liposomes made of a mixture of phosphatidylcholine and cholesterol. The reconstituted liposomes were interfaced with capacitance sensors developed at the Applied Physics Laboratory of The Johns Hopkins University. The capacitance, measured by a GR-1657-RLC Digibridge, increased when the biosensor was exposed to ACh in a dose-dependent and reversible manner. A d-tubocurare-treated biosensor failed to respond to ACh. Effects of anticholinesterases on the ACh receptor-based biosensor will be reported.

CERTAIN MUSCARINIC ANTAGONISTS ARE NONCOMPETITIV INHIBITORS OF THE NICOTINIC ACETYLCHOLINE RECEPTOR.

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The muscarinic antagonists, atropine, aprophen and benactyzine may be used as antidotes for the treatment of organophosphorus (OP) poisoning. We have studied the interaction of these drugs, focusing mainly on aprophen and benactyzine, with nicotinic acetylcholine receptor (AChR) in BC3H-1 intact muscle cells and with receptor-enriched membranes of Torpedo californica. Aprophen and benactyzine diminish the the maximal carbamylcholine (carb)-elicited sodium influx into muscle cells without shifting K_{act} (carb concentration eliciting 50% of the maximal ^{22}Na influx). The concentration dependence for the inhibition of sodium influx by aprophen and benactyzine occurs at lower concentrations (K_{ant} = 3 and 50μM, respectively), than those needed for the displacement of [^{125}I]-α-bungarotoxin from the agonist/antagonist sites of AChR (K_B = 83 and 800μM, respectively). The effective concentration for the inhibition of AChR response by atropine (K_{ant} = 150μM) is significantly higher than those obtained for aprophen and benactyzine. Both aprophen and benactyzine interact with AChR in its desensitized state in BC3H-1 cells without further enhancing agonist affinity. Furthermore, in BC3H-1 muscle cells, aprophen and benactyzine do not alter K_{des} (equilibrium concentration of carb which diminshes 50% of the maximal response). However, both compounds preferentially associate with the high-affinity state rather than the resting state of Torpedo AChR. The K_d values obtained for aprophen and benactyzine from equilibrium displacement of [^3H]PCP in Torpedo are: 16.4 and 384μM, compared to 0.7 and 28.0μM, in the presence of carb, respectively. These data imply that aprophen and benactyzine binding to Torpedo AChR is allosterically regulated by the agonist site.

While previous studies have shown that aprophen and benactyzine are muscarinic antagonists at 10-100nM, the present study demonstrates that these ligands are effective noncompetitive inhibitors of AChR at 3-50μM, in either Torpedo or mammalian muscle cells. These effective concentrations correspond very well with the plasma level of these drugs found in-vivo to produce a therapeutic response against OP poisoning.

BINDING PROPERTIES AND TRANSMEMBRANE SIGNALLING OF MUSCARINIC RECEPTOR SUBTYPES

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The existence of various muscarinic receptor subytpes in the central nervous system is indicated by multiaffinity binding curves for agonists such as carbachol (CCh) and by differential changes of the binding properties of muscar·nic receptors, from various brain regions, in response to chemical treatments such as N-ethylmaleimide. However, it is unclear whether these various receptor subtypes use different transmembrane signalling mechanisms to achieve the physiological response.

In the present work we used a novel selective agonist, cis-2-methylspiro(1,3-oxathiolane-5,3')quinuclidine (AF102B), designed and synthesized at our laboratory, as a tool to study the transduction mechanism of the M1 receptor subtype. The binding properties and the biochemical effects of this agonist were compared to those of the ganglionic M1-selective agonist, McN-A-343 and to oxotremorine which has a relative high selectivity toward M2 receptor subtype. The potency of AF102B in displacing the nonselective antagonist ^{3}H-QNB from cerebellar homogenate (rich in M2 receptor subytpe) was much lower than that of oxotremorine. However AF102B was similar to McN-A-343 in displacing ^{3}H-QNB from forebrain homogenate (rich in M1 receptor subtype). These data indicated the relative M1-selectivity of AF102B. Displacements of the selective M1 antagonist, ^{3}H-pirenzepine and the nonselective agonist, ^{3}H-cis-methyl-dioxolane, also showed that AF102B was the most selective M1 compound when compared to oxotremorine, McN-A-343, AF102A (the trans isomer) cis-AF30 and trans-AF30 (the dioxolane analogs, respectively). The agonistic nature of AF102B was shown in binding studies, where nonhydrolyzable GTP analogs, as in case of CCh, shifted the displacement curve to the right, as well as in functional studies where AF102B, same as CCh, was able to induce atropine-sensitive contractions of smooth muscles (isolated guinea-pig ileum and trachea). However, unlike the agonist CCh, AF102B affected neither the adenylate cyclase activity nor did it potentiate phosphoinositide (PI) turnover. Nevertheless, AF102B, inhibited the CCh-induced hydrolysis of phosphatidylinositol bisphosphate but did not affect the CCh-induced inhibition of adenylate cyclase. Thus it seems that although activation of M1 receptors in the brain does not potentiate PI turnover, this biochemical function is somehow associated with the M1 receptor subtype. Indeed, the distribution of M1 subtypes receptors in the various brain regions is similar with that of CCh-induced PI turnover (e.g. cortex and striatum), while the distribution of M2 subtypes receptors is similar with that of CCh-induced inhibition of adenylate cyclase (e.g. cerebellum).

BIOCHEMICAL AND BEHAVIORAL EFFECTS OF ACETYLCHOLINESTERASE INHIBITION IN BRAIN

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The effect of acute (single or multiple dosage) and chronic (up to 50 days) administration of three drugs producing reversible inhibition of acetylcholinesterase (AChE) in brain (physostigmine, tetrahydroaminoacridine and metrifonate) was compared in rats. The results reveal major differences in biochemical effects such as percent and duration of AChE inhibition, regulation of acetylcholine synthesis and release, uptake of choline and binding to cholinergic receptors. Behavioral effects such as "stereotypy" and tolerance are also markedly different. These results suggest differences in mechanism of action of various AChE inhibitors in brain. These experimental findings have potential clinical implications for the symptomatic therapy of Alzheimer patients, suggesting new strategies and novel models of administration of cholinesterase inhibitors. (Supported by grants from the Natl. Inst. Aging #AG05416-01A1 and Illinois Dept. Public Health).

MOLECULAR BASIS OF ANTICHOLINESTERASE ACTIONS ON NICOTINIC AND GLUTAMATERGIC SYNAPSES.

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The understanding of the interactions of cholinesterase (ChE) inhibitors, including certain insecticides, and the ChE-reactivating oximes with the macromolecular entity comprising the nicotinic receptor and its ion channel (AChR) is of fundamental importance for the proper treatment of insecticide poisoning and sel tion of appropriate therapeutic approaches to central cholinergic disorders. In addition to their ChE-inhibitor properties, both reversible and irr versible anticholinesterase agents, (carbamates and organophosphorus compoun) have direct actions on the peripheral nicotinic AChR. acting as agonists, blocking the ion channel in its open conformation via noncompetitive mechanisms or enhancing receptor desensitization. The pyridinium compounds with oxime groups, such as 2-PAM and HI-6, and another bispyridinium compound which lacks the oxime group, namely SAD-128, have been shown in our laboratory to modulate the ionic currents occurring at the frog endplate region. Voltage-clamped endplate currents (EPCs) are increased in peak amplitude and prolonged by ChE inhibition, but EPCs were decreased in amplitude and either shortened or converted into biphasic decays in voltage-dependent manner by the noncompetitive actions of 2-PAM, HI-6 and SAD-128. Acetylcholine (ACh)-induced channel openings (open times) were also shortened by HI-6 in a voltage-dependent manner as vealed by patch clamp studies. The AChR channel-blocking effect of these pyridinium compounds may remain as one of the mechanisms by which they produce antidotal effects against organophosphate agents. The strength of this hypothesis is further enhanced by the significant protection against lethality of organophosphorus agents offered by (+)physos igmine, which in the absence of significant reversible ChE hibition may be attributed to a noncompetitive blockade of AChR. Other aspects of anti-ChE agents independent of ChE inhibition are also under investigation. Furthermore, studies with forskolin and its analogs have disclosed a possible involvement of phosphorylation in the regulation of nicotinic AChR activation. Ion channel blockade produced by nicotinic noncompetitive antagonists at the locust glutamatergic synapse raises the possibility of certain similarities between ACh and glutamate receptors.

The biochemical and electrophysiological aspects of the central nervous system nicotinic AChR are being investigated using selective and stereospecific agents. Although sc differences in agonist effects at entral and periphe

receptors have been encountered, the present study disclosed
marked homology between the putative hippocampal AChRs and
those on the muscle. In binding experiments, (+)anatoxin-a
(Mol. Pharmacol. 29: 250. 1986) was a potent competitor of
[^{3}H](-)nicotine binding to rat brain membranes (K$_i$ - 3 x
10^{-10}M); the (-)anatoxin enantiomer was two orders of mag-
nitude less effective. To see if (+)anatoxin-a also acts as
an agonist in the CNS, the toxin was tested in a model system
based on the presynaptic, nicotinic facilitation of trans-
mitter release. Synaptosomes from rat hippocampus were
loaded with [^{3}H]choline so that perfusion with Kreb's
bicarbonate buffer into which pulses (100 μl; 50 μM) of
(-)nicotine were introduced resulted in the release of
discrete peaks of [^{3}H]ACh. This release of neurotransmitter
could be partially blocked by the nicotinic antagonist
dihydroβerythroidine (10 μM). Similarly, hippocampal
synaptosomes loaded with [^{3}H]GABA responded to pulses of
nicotinic agonist by releasing this radiolabelled trans-
mitter. The higher labelling [^{3}H]GABA allowed lower agonist
concentrations to be tested. In this case, 10 μM (-)nicotine
and 1 μM (+)anatoxin-a released comparable amounts of
[^{3}H]GABA. The results suggest that (+)anatoxin-a is at least
5 times more effective than (-)nicotine at presynaptic
nicotinic receptors in mammalian brain. (D.R.E. Macallan,
G.G. Lunt, S. Wonnacott, Y. Aracava, M. Rapoport and E.X.
Albuquerque, Proc. Nat. Acad. Sci. U.S.A., submitted). The
hippocampal AChRs showed a similar sensitivity to histrioni-
cotoxin, a probe for ion channel sites, as the muscle AChRs
(FEBS Lett. 212:292, 1987). Using (+)anatoxin-a and ACh as
probes of nicotinic AChR and employing the patch-clamp
technique, we recorded channel activation in hippocampal
neurons cultured from fetal rats (10 to 20-day-old culture).
Channel activity was recorded from an area close to the axon
hillock of the hippocampal neurons. Both ACh (0.4 μM) and
(+)anatoxin-a (0.1-0.4 μM) activated single channel currents
whose conductances and durations were quite similar to those
recorded at the perijunctional region of the muscle endplate.
Additionally, AChRs activated by either ACh or (+)anatoxin-a
were sensitive to α-bungarotoxin and dihydroβerythroidine.
These studies demonstrate the presence of functional
nicotinic AChRs on the neurons of rat hippocampus. The
sensitivity of central nicotinic receptors to (+)-anatoxin-a
and analogues and physostigmine has also been tested using
the patch-clamp technique in isolated hippocampal pyramidal
cells, ganglion cells of the retina and brain stem
respiratory neurons. This study may provide more efficacious
and specific analogues for further biochemical and electro-
physiological analyses of various receptors, including those
involved in cholinergic disorders.

ROLE OF LIPIDS IN THE REGULATION OF THE BINDING PROPERTIES OF RAT HEART MUSCARINIC RECEPTORS AND THEIR INTERACTION WITH G-PROTEINS

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In order to investigate the dependence of the properties of
cardiac muscarinic receptors, and especially their interactions
with G-proteins, on the membrane lipid composition, we employed
primary myocyte cultures prepared from the hearts of newborn
rats. The membrane lipid composition in these cells undergoes
dramatic changes with the culture's age (number of days in
culture), and it can also be manipulated by treatment with
liposomes of specific compositions. The changes in the lipid
composition resulted in significant alterations in the
organisation of the plasma membrane lipids in lateral domains.
Concomitant alterations were observed in several properties of
the muscarinic receptors. Aging of the cells in culture resulted
in reduced muscarinic binding capacity, and in changes in the
proportion of high and low affinity states towards agonists. In
parallel, major changes were observed in the coupling of the
muscarinic receptors with G-proteins: while guanine nucleotides
converted high affinity agonist binding sites to the low affinity
form in homogenates of young cultures, a reverse effect
(conversion from low to high affinity sites) was observed in aged
cultures. These alterations in the properties of the muscarinic
receptors appear to be intimately associated with the age-
dependent alterations in cellular lipid composition and
organisation, since manipulations of the lipid composition of
aged cultures back to that of young cultures by treatments with
phosphatidylcholine-containing liposomes returned all the
muscarinic parameters measured back to those observed in young
cultures.

NEUROTRANSMISSION AND SECOND MESSENGERS IN XENOPUS OOCYTES

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Xenopus oocytes present a convenient experimental
system for the study of second messenger-mediated
neurotransmitter responses. These cells display a
muscarinic response, consisting of a two component Cl
current, accompanied by Cl current fluctuations and a
less prominent slow K current. The muscarinic response
is most reliably observed in intact follicles (oocytes
that still retain enveloping support tissues). The
muscarinic Cl current response is mimicked by
intracellular injection of Ca or IP3. It is blocked by
intracellularly injected EGTA. Application of ACh
results in increased polyphosphoinositide breakdown,
enhanced ^{45}Ca efflux from preloaded cells, and
inhibition of the response to adenosine, probably
through the activation of protein kinase C. Thus, the
muscarinic membrane response (Cl current) in the oocyte
is mediated throughs the phospholipase C - Ca
mobilization pathway.

When Xenopus oocytes are injected with exogenous
messenger RNA, they express membrane receptors and ion
channels characteristic of the source tissue from which
mRNA was extracted. This approach allows the study of
ion channels and neurotransmitter responses in a
standrad membrane environment. In addition, since
oocytes are large (> 1 mm in diameter) cells, one can
employ techniques, such as double electrode voltage
clamp and intracellular injections, that are impossible
in the source tissues.

In Xenopus oocytes, elevation of internal Ca causes an
increase in Cl conductance. We further characterized
this conductance using the ionophore A23187. At 0.25-1
mM, Ca evoked an inward current that reached a plateau
in about 10 s., and declined gradually within many
minutes in the presence of Ca, but within seconds upon
washout of Ca. At higher concentrations, Ca caused a
two component Cl current response, with the appearance
of a fast component. The fast component was more
sensitive to 1-4 mM 9-antracene carboxylic acid (9 AC)
blocker. Exposure of the cell to low (0.1-0.2 mM) Ca
concentration for several minutes greatly diminished
the amplitude of Cl current evoked by a subsequent
application of a higher dose of Ca, suggesting that the
inactivation of the Cl current was Ca-dependent.

Shallow, submembranl injections of IP3 in the animal
hemisphere of the oocyte evoked a two-component
response comprised of a rapid transient component
followed by a slow sustained component. When the
injection pipette was further inserted into the cell
(to 300 um below the cell membrane), the fast component
diminished and the slow component remained unchanged or
even increased. Similar two-component response was
obtained by the injection of a single large dose (20-30
pmol) of $CaCl_2$.

Cardiac voltage dependent Ca channels (VDCC) appeared
in the oocyte following injection of mRNA obtained from
the rat heart. Thus, the oocyte may serve as a useful
model for the study of Ca channel formation and
modulation.

STRUCTURAL STUDIES AIMED AT UNDERSTANDING THE FUNCTION OF THE ACETYLCHOLINE RECEPTOR

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The three-dimensional structure of the acetylcholine
receptor has been determined for the first time in the
absence of attached cytoskeletal components. This permits
a difference map which identifies loci of attachment of
43 kD cytoskeletal proteins. The distribution of
molecular mass shows that there is little of the protein
which is ordered on the cytoplasmic side of the plasma
membrane.

The topology of chains within subunits has been
mapped by antibody binding to native tissue. Antibody
experiments on subcellular fractions which utilize
detergent or small amphiphiles are prone to serious error
since these treatments can undo regions of protein on one
side of the membrane selectively and so may give
incorrect assignments of location. Results of proteolytic
cleavage lead to assignments of other regions as they
emerge from the plasma membrane and thus define important
aspects of the folding of this molecule.

The status of amphipathic helices and their
contribution to channel formation is discussed alongside
experiments with a bacterial channel-forming protein
whose structure is being worked out by x-ray
crystallography. The direct answer to structure of the
ACh receptor will come from x-ray studies of three-
dimensional crystals. Progress in understanding the
x-ray diffraction from crystals grown in our laboratory
is discussed. Effects of lipids, detergents, and their
ratios upon crystallization seem critical for the
subsequent crystallization of affinity-purified protein.
Effects of cholesterol and local anaesthetics on receptor
conformation and stability are discussed since they bear
directly on issues concerned with functional
reconstitution of ACh receptor.

The origin of gating in the ACh receptor is
discussed in light of studies using terbium/calcium
exchange. Evidence towards an electrostatic gate is
presented.

Antibodies as Probes of the Structure and Function of the Nicotinic Acetylcholine Receptor

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1. Antibodies were raised against eight synthetic peptides matching in sequence preselected stretches of the aminoacid sequence of the nicotinic acetylcholine receptor (nAChR) from *Torpedo marmorata*. To avoid ambiguity as to the exact sequence location of the antibody epitopes, synthetic peptides of only 5-7 aminoacids in length were employed. The results of epitope mapping support a model with three transmembrane domains, one membrane associated α-helical domain and extensive β-structure at the extracellular and cytoplasmatic surfaces of the receptor molecule.

2. Monoclonal antibodies raised against the native receptor protein were employed to study the functional organization of *Torpedo* nAChR. We report on antibodies (i) competing with selected groups of cholinergic ligands for binding to the nAChR, (ii) acting as allosteric ligands, (iii) blocking the agonist-induced cation flux into *Torpedo* membrane vesicles, (iv) blocking agonist-induced single channel activity in embryonic muscle cells, and (v) interfering with desensitization of the nAChR-integral ion channel.

THE HELIX-II MODEL OF THE ION CHANNEL OF THE NICOTINIC
ACETYLCHOLINE RECEPTOR

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A model of the ion channel of the nicotinic acetylcholine receptor
is proposed, picturing the channel as being formed by the transmembrane
Helix M2. The following features of the channel will be discussed:
Channel entrance (extracellular) 30 Angstrom diameter; constant diameter
down to level of lipid membrane; funnel-shaped transmembrane portion
lined by Helix M2; binding site of channel blockers (ser 262 in delta
chain) close to intracellular exit of channel. There too the narrowest
part and the selectivity filter having a diameter of 6.4 Angstrom.
Upper part of channel hydrophobic, selectivity filter polar. Channel
filled with water. Experimental support for this model from this and
other laboratories will be discussed.

CORRELATING STRUCTURE WITH FUNCTION OF THE CHOLINERGIC BINDING SITE

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The nicotinic acetylcholine receptor (AChR) has been subjected to extensive experimental scrutiny and indeed much has been learned about this multimeric protein complex. However, in spite of the impressive amount of data that has accumulated we still can not explain the mechanisms responsible for its action. One approach which has been adopted in our laboratory has been to focus on a single, well defined functional domain of the receptor and systematically analyse it with the intent to correlate its structure with its function. Thus, over the past few years, we have been studying the cholinergic binding site.

Karlin and his co-workers demonstrated that the ligand binding-site is situated 1nm away from a sensitive disulfide. As a result and with the appearance of the complete amino acid sequence of the α-subunit, models quickly appeared placing the binding-site in the vicinity of cysteine residues postulated to be extracellular. Biochemical analyses have allowed the mapping of the binding-site to the area of cys-192/cys-193. This has been accomplished by the protein blot analysis of proteolysed α-subunits probed with sequence specific antibodies. Furthermore, cys-192 was found by Karlin to be that which is affinity alkylated and is apparently disulfide linked to cys-193.

As much attention has been placed on the cysteine residues related to ligand binding, it was of interest to establish the disulfide arrangement of the α-subunit. For this native receptors have been selectively alkylated with 3-(N-maleimido-propionyl)biocytin (MPB). The α-subunits of the various biontinylated receptors were isolated, digested with V8-protease and blotted. The blots were probed with ^{125}I-labeled-avidin revealing those fragments which contain modified cysteines. It was found that three disulfides exist: cys-128 to cys-142; cys-192 to cys-193; cys-412 to cys-418, and that cys-222 is unpaired.

In order to investigate the specific contributions of the various residues which lie in the region of the binding-site, genetically engineered binding-sites have been produced that continue to bind cholinergic ligands. Synthetic oligonucleotides corresponding to the amino acid sequence α-184-200 of *T. californica* AChR have been cloned using the bacterial expression vector pATH2. Such transformed cells can be induced for the selective expression of the *trpE* fusion-protein which contains the AChR sequence at its COOH-terminus. These fusion-proteins can be highly enriched and have been analysed for their capacity to bind cholinergic ligands. Thus α-184-200 binds α-bungarotoxin with an affininty of 10^{-7} M. Tubocurarine competes this binding with an IC_{50} of 10^{-4} M and carbamylcholine competes with an IC_{50} of 10^{-2} M. The bacterially expressed binding sites are being subjected to mutagenesis so to evaluate the involvement of individual residues in the binding process.

In addition, we have engaged in a survey of naturally occuring variants of the ligand binding-site. Two biological systems are being analysed. Genomic libraries have been prepared for the snake, cobra and the moth *Spodoptera littoralis* and are currently being screened for α-subunit homologues.

STUDIES OF THE BINDING OF AGONISTS TO THE ACETYLCHOLINE RECEPTOR

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The nicotinic acetylcholine receptor (AChR) is a membrane protein
t..at changes the permeability of the postsynaptic membrane to sodium
ions, following binding of the eurotransmitter acetylcholine. Direct
NMR study of the AChR is made difficult by its high molecular weight
and the requirement of detergents for its solubilization. We have shown
that the binding of agonists to the AChR may be studied by two-dimensio-
nal nuclear Overhauser effect (2-D NOE) and measurement of selective T_1
(T_1^{sel}) of the agonist protons.

It was pointed out a few years ago that T_1^{sel} is very sensitive to
the binding of ligands to large macromolecules[1]. Indeed, significant
shortening of the T_1^{sel} of acetylcholine, carbamylcholine and nicotine
was found in the presence of solubilized AChR from the Torpedo Califor-
nica electric tissue. The effect was abolished by the addition of α-
bungarotoxin which binds very tightly to the receptor. T_1^{sel} can also be
used for the determination of binding constants of ligands[2]. Since
binding constants of agonists to the AChR are very large, only relative
binding constants could be obtained, by competition experiments. Thus,
by following T_1^{sel} of the nicotine aromatic protons as a function of the
concentrations of other agonists, relative binding constants of
1 : 7 : 0.2 : 0.04 were found for nicotine, acetylcholine, carbamylcho-
line and muscarine, respectively.

2-D NOE experiments on nicotine in the presence of AChR yielded

negative cross-peaks corresponding to NOE's between the various protons of the bound agonist as well as NOE's due to transfer of magnetization from the receptor to the agonist. A combination of the NOE and T_1^{sel} data allows us to calculate the distances[3] between the protons of the agonist in the bound state, from which the conformation of the bound agonist may be derived. Those groups on the AChR which are in close contact with the agonist are being identified on the basis of transferred NOE from the AChR to the agonist protons.

(1) G. Valensin, T. Kushnir and G. Navon, J.Mag.Reson. **46**, 23 (1982).

(2) T. Kushnir and G. Navon, Bull.Magn.Reson. **6**, 50 (1984).

(3) D. Kaplan and G. Navon, J.Chem.Soc. Perkin Trans.II **10**, 1374 (1981).

EXPRESSION OF FUNCTIONAL NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS FROM CLONED cDNA SEQUENCES

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A family of genes coding for proteins homologous to the muscle nicotinic acetylcholine receptor α-subunit has been identified in the rat genome. Three members of this family, alpha2, alpha3, and alpha4 genes are transcribed in the central and peripheral nervous systems in areas known to contain functional nicotinic receptors. We have identified an additional gene, beta2, which encodes a protein homologous to the α-subunits but which lacks the cysteine residues thought to be close to the ligand binding site. The beta2 gene encodes a protein which, in combination with either the alpha2, alpha3, or alpha4 proteins will form functional nicotinic acetylcholine receptors in *Xenopus* oocytes. Oocytes expressing either alpha2, alpha3, or alpha4 protein in combination with the beta2 protein produced a strong response to acetylcholine. Oocytes expressing only the alpha4 protein gave a weak response to acetylcholine. These receptors are activated by both acetylcholine and nicotine. They are not blocked by alpha-bungarotoxin, which blocks the muscle nicotinic acetylcholine receptor. Thus, the receptors formed by the alpha2, alpha3, alpha4, and beta2 subunits are pharmacologically similar to the ganglionic type neuronal nicotinic acetylcholine receptor. These results demonstrate that the alpha2, alpha3, alpha4 and beta2 genes code for functional nicotinic acetylcholine receptor subunits which are expressed in the brain and peripheral nervous systems.

SINGLE-CHANNEL AND LIGHT-FLASH MEASUREMENTS ON MOUSE-*TORPEDO* ACETYLCHOLINE RECEPTOR HYBRIDS EXPRESSED IN *XENOPUS* OOCYTES. Lei Yu, Reid J. Leonard, Cesar Labarca, Henry A. Lester. Division of Biology, Caltech, Pasadena CA 91125, USA

This work extends previous studies on equilibrium properties of receptors expressed in oocytes by injection of SP6 RNA. We have examined voltage sensitivity at the level of single-channel recording with several hybrid receptors: $\alpha_M\beta_M\gamma_M\delta_M$, $\alpha_M\beta_T\gamma_M\delta_M$, $\alpha_M\beta_M\gamma_M\delta_M$, $\alpha_M\beta_T\gamma_T\delta_T$, $\alpha_T\beta_T\gamma_T\delta_M$, and $\alpha_T\beta_T\gamma_T\delta_T$. In outside-out patches, all combinations showed a linear single-channel current-voltage relationship; but open duration depended on voltage. Average channel duration, τ, was measured at -60 mV and +60 mV. The extent of the voltage dependence of each combination was estimated by the ratio of $\tau_{-60mV}/\tau_{+60mV}$, and the results were in agreement with our previous conclusions from the macroscopic measurements. Closed times had less than 1/5 the voltage sensitivity of the open times. Thus, of the three possible parameters underlying voltage sensitivity (channel conductance, opening rate, and closing rate), the latter makes the major contribution to the voltage sensitivity of the macroscopic ACh-induced conductance.

In an attempt to identify portions of the subunits' structure that may be involved in formation of the ion channel of the AChR, we are also comparing the effects of local anesthetics on the response of hybrid AChRs. The data show that some hybrids differing in mean open time in the absence of local anesthetic exhibit very similar open times in the presence of the blocker QX-222, thus indicating that the forward blocking rate is only weakly influenced by subunit composition. The flash-activated channel blocker, EW-1, is also being used with macroscopic agonist-induced currents to provide efficient screening of the blocking/unblocking kinetics. Results show that the β subunit strongly influences the pharmacological properties of the *cis* and *trans* isomers. The *cis* isomer is a potent channel blocker in all-mouse receptors and in several other hybrids; for these receptors, effects of EW-1 resemble those previously reported for *Electrophorus* electroplaques. However, the *trans* isomer is also a potent blocker in those hybrids containing the *Torpedo* β subunit. Sponsored by NS-11756 and MDA (fellowship to R.J.L. and grant).

ION CONDUCTANCE OF GENETICALLY MODIFIED ACETYLCHOLINE RECEPTOR CHANNELS

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Functional nicotinic Acetylcholine Receptor (AChR) channels are synthesized and inserted into the plasma membrane of Xenopus oocytes following th microinjection of mRNA extracted from tissues that express the AChR protein. Injection of specific mRNAs, obtained by in vitro transcription from cloned cDNAs that encode the AChR subunits, leads to a high channel density in the oocyte membrane so that single AChR channels can easily be investigated with the patch clamp technique. Their conductance and gating behavior closely agree with those of native AChR channels studied in the neuromuscular endpl te or in cultured muscle cells.

The AChR proteins are oligomers composed of four different peptide subunits. Therefore, the four corresponding mRNAs must be injected simultaneously into oocytes to obtain proper AChR channels. However, by deleting subunits or by exchanging subunits from the AChRs of different species, modified AChR channels may be synthesized whose properties illuminate the role played by different subunits in receptor and channel function.

With modern methods of genetic technology, the cDNA coding for an AChR subunit can be modified directly and specifically. and then transcribed in vitro to obtain mRNA encoding a mutated AChR subunit. This, when injected into Xenopus oocytes together with the other required subunits, can direct the synthesis of novel mutant AChR channels with known primary structure, whose function can then be studied in detail with patch recording. For example, the delta subunits of bovine and Torpedo AChR differ in their primary sequence and in several of the properties that they confer to the complete receptor channel complex. By producing chimaeric delta subunits and incorporating them into AChR channels in oocytes, some of the functic al differences between the two parental subunits could be assigned to specific locations on the prim ry sequence. Thus, the M2 segment of the channel has been asso atad with the control of ion permeation. Similarly, the effect on channel function of a single specific point mutation at practically any desired position along the primary sequence can be studied.

The permeation of ions through the open AChR channel has been studied for a number of native, chimaeric and modified AChR types. The conductance, the i-V relationship and the effects of divalent ions are affected by some of the differences in primary structure between the various kinds of AChR. Therefore, such experiments can illuminate the relation between channel structure and function.

However, since there is no definitive three-dimensional structure available for the AChR as yet, it is necessary to devise models of ion permeation that can assist in the interpretation of these results. Numerous such models of various complex ty can be found in the literature.

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It was found that a quite simple model, consisting of a two-barrier single-well Eyring rate-limited pore together with the Graham-McLaughlin description of the effects of surface charge, is quite satisfactory to represent the permeation of ions through AChR channels. An important feature of the model is that the central energy well within the channel is not necessarily a binding site. Instead, the saturation of channel conductance arises from the accumulation of permeant ions by surface charges near the channel pore. The shielding of surface charges also predicts the observed effects of divalent ions on channel conductance. The numbers of charged amino acid residues near the M2 segments of various AChR subunits are found to correlate with the observed conductance behavior as suggested by the model.

RECEPTORS FOR ACETYLCHOLINE IN THE NERVOUS SYSTEM OF INSECTS

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The central nervous system of arthopods is highly cholinergic in-
nervated; thus insect ganglia appear most appropriate for studying
neuronal acetylcholine receptors (AChR). Using specific ligands,
high concentrations of cholinergic binding sites have been detec-
ted; in contrast to the vertebrate brain, the nicotinic receptor
type predominates in insects, whereas only a small portion dis-
played muscarinic properties. Subtypes of muscarinic receptors
could be distinguished; the M_2 type appears to be located at nerve
terminals and to be involved in the feedback regulation of acetyl-
choline release.
Attempts were made to identify the nicotinic acetylcholine recep-
tor from locust nervous tissue. A large complex receptor protein
has been purified which appeared to be composed of 4-5 identical
or very similar polypeptides. When reconstituted in planar lipid
bilayers, the native protein gave a functional ion translocating
system. Agonists such as acetylcholine, carbamylcholine and sube-
ryldichoiine induced fluctuations of single channels which were
blocked by d-tubocurarine, suggesting that the protein represents
a functional cholinergic receptor channel. The channel was selec-
tively permeable for monovalent cations but was impermeable for
anions. The conductance of the channel (75 pS in 100 mM NaCl) was
independent from the type of agonist used. Kinetic analysis of the
channel gating revealed that at high agonist concentration multi-
ple gating events and bursting appeared. Approaches to determine
the cooperativity of channel activation showed that the nicotinic
acetylcholine receptor from nerve cells of insects is apparently
activated by only one agonist molecule. Immunological approaches
revealed that the receptors are localized in the neuropile and
that there are obviously significant molecular similarities bet-
ween the constituents of the neuronal insect receptor and the pe-
ripheral heterooligomeric vertebrate receptor. This was confirmed
when the N-terminal amino acid sequence of an insect receptor po-
lypeptide fragment was determined. As a first step towards an ap-
plication of recombinant DNA techniques, RNA preparations from lo-
cust nervous tissue were probed for receptor specific mRNA using
reticulocytes and Xenopus oocytes as expression systems. Oocytes
microinjected with insect $polyA^+$-RNA produced neuronal AChR poly-
peptides, which were inserted into the oocyte surface membrane and
displayed specific binding of α toxins. Ion flux studies have pro-
vided evidences that the binding sites represent indeed ACh-gated
ion channels, functional AChRs.

The membrane environment of the nicotinic acetylcholine receptor
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Detailed knowledge of the membrane framework surrounding the
nicotinic acetylcholine receptor (AChR) is key to understanding
its structure, dynamics and function. Theoretical models based on
the application of thermodynamic criteria to the known primary
sequence of the AChR polypeptide chains assign portions of these
to the bilayer region. The nature of the protein-lipid
interactions occurring in the bilayer is not known, nor is the
structural relationship between the two moieties.
The composition of the lipids in which the transmembrane AChR
chains are inserted has been characterized in three *Torpedinidae*
species. The marked unsaturation of the major phospholipids found
may explain some of the physical properties of the bulk lipid in
AChR-rich membranes; more than 50% of the fatty acids are long
chain polyunsaturates. Docosahexanoate makes up more than 70% of
the latter in *T. marmorata*. Phospholipid classes and fatty acyl
chains bear considerable resemblance among species, perhaps
providing this evolutionarily conserved protein with a similarly
constant milieu for its optimal functioning.
Changes in physical properties of the AChR membrane are known to
follow on from extraction of non-receptor, peripheral proteins.
These changes may bear relationship to the recently found
selective depletion of certain phospholipid classes which
accompanies peripheral protein extraction.
Metabolic studies conducted both *in vivo* and *in vitro* using
radiolabelled precursors indicate the presence of active pools
among membrane phospholipids, particularly in the minority
polyphosphoinositides.
Studies aimed at defining the topography of lipids in the
membrane show the asymmetrical distribution of phospholipid
classes between the two half-layers. Although we still ignore
which lipids are in the immediate microenvironment of the AChR,
ESR studies reveal the presence of an annulus of relatively
immobilized lipid, consisting of about 40 phospholipid molecules
per AChR monomer, in active exchange with the bulk bilayer lipid.
Lateral diffusion of lipid in the plane of the membrane appears
to be a free process as measured by fluorescence anisotropy
decay.

BIOPHYSICAL STUDIES OF ACETYLCHOLINE RECEPTOR IN RECONSTITUTED
MEMBRANES: ROLE OF LIPIDS IN REGULATING FUNCTION

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Nicotinic acetylcholine receptor (AChR) from the electric tissue
of the electric ray Torpedo californica has been purified by
affinity chromatography in cholate solution and reincorporated
into membranes of defined lipid composition by dialysis. The
interactions of the AChR with lipids have been examined by several
different biophysical techniques including fluorescence quenching,
EPR-spin labelling, and FTIR spectroscopy. Important lipid-
protein interactions detected by the biophysical studies have been
correlated with the functional effects of the lipids on both the
ligand binding and ion permeability control properties of the
AChR.

Using a variety of different lipid mixtures, it was determined
that the low-to-high binding affinity transition for activating
ligands requires an optimal membrane fluidity, as measured by the
order parameter of a fatty acid spin label incorporated into
reconstituted membranes. Membranes that had either too high or
too low a value for the order parameter were unable to undergo the
transitions induced by carbamylcholine even though they could bind
alpha-bungarotoxin. The effects were reversible as judged by
temperature dependence and re-reconstitution into the proper lipid
environment. Receptor-mediated ion permeability also required an
optimal fluidity since the affinity transition appears to be a
necessary, but not sufficient, condition for functional receptors.
However, the ion permeability properties also showed a requirement
for specific lipids under the reconstitution conditions used.
Only those lipid mixtures containing cholesterol and negatively-
charged lipids were active in ion flux assays.

In parallel studies, the effects of different lipid mixtures on
the overall conformation of the AChR were analyzed by FTIR
spectroscopy. Cholesterol appeared to increase the average alpha
helix content of the AChR as monitored by an increased intensity
at 931 cm-1 and negatively charged lipids appeared to increase the
beta sheet content as monitored by increased spectral intensity at
988 cm-1. The combined increases in both parameters correlate

well with the lipid requirements for functional ion channels. The
results suggest that cholesterol, or related analogs, may
stabilize alpha helical structures associated with the ion
channel.

A special role for cholesterol in AChR structure and function has
also been revealed by fluorescence quenching studies using
brominated analogs of phosphatidylcholine and cholesterol. The
brominated analogs partially quench the intrinsic fluorescence of
the AChR by a static mechanism. Competition betwen brominated and
non-brominated lipids provides quantitative data that can be used
to calculate relative binding constants for different lipids to
the membrane-surface areas of the receptor. In the case of
cholesterol, additional binding sites on the protein that cannot
be displaced by phospholipids are detected. These sites may
represent regions of the protein that have a relatively high
affinity for cholesterol. Experiments are in progress to
determine if the extra cholesterol binding sites are related to
the role of cholesterol in stabilizing alpha helical structures
and in providing an appropriate lipid environment for ion channel
activity in reconstituted membranes.

NEW APPROACHES TO STUDIES OF NEURONAL RECEPTORS

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During the past years, 40 chemical signals (neurotransmitters) and
a variety of different receptor proteins responsible for the
transmission of signals between nerve cells and between nerve and muscle
cells have been identified in electrophysiological experiments.
Investigations of the relationship between the signal transmission
process and the function and structure of the receptors have been
hampered by the time resolution of the available chemical kinetic
techniques, and by the availability of receptor proteins in sufficient
quantities for the structure to be determined. Three different
approaches adopted in this laboratory to overcome the problem will be
discussed.
(a) Two kinetic techniques have been developed that allow one to measure
the ligand-binding steps, channel-opening steps, and receptor
desensitization (inactivation) over a wide concentration range of
neurotransmitter and inhibitors, directly on a cell surface: (i) a cell
flow method with a 5 ms time resolution, and (ii) synthesis of inactive
neurotransmitter precursors, which can be equilibrated with receptors on
cell surfaces and then photolyzed to the active neurotransmitter with $t_{\frac{1}{2}}$
of 50 µs.
(b) Yeast can be grown in large quantities. We have shown that it is
capable of synthesizing the acetylcholine receptor subunits and
inserting them into its plasma membrane, and we are studying the
structural and functional properties of the polypeptide chains. The
expectations are that large enough quantities of a variety of receptor
proteins can be produced and assembled by yeast for their structure and
function to be studied.

THE BINDING SITE AND PHOSPHORYLATION SITES OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

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The nicotinic acetylcholine receptor (AChR) is a neurotransmitter-regulated ion channel which mediates synaptic transmission at the post-synaptic membrane of the neuromuscular junction. The purified AChR is a 250 kDa transmembrane glycoprotein and consists of four subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$. Synthetic peptides corresponding to sequences from the AChR and their respective antibodies, are being employed for mapping and analysing the cholinergic binding site and phosphorylation sites of the receptor. We have previously shown that a synthetic dodecapeptide corresponding to residues 185-196 of Torpedo AChR α-subunit, contains the essential elements for α-bungarotoxin (α-BTX) binding. By quantitative analysis of the cholinergic binding properties of this peptide we have demonstrated that this dodecapeptide also includes the neurotransmitter binding site. Synthetic peptides corresponding to residues 185-196 of human and mouse AChR α-subunits are also being analysed. These studies along with specific chemical modifications point at the importance of the cysteine and tryptophan residues for the binding. We are now attempting to elucidate the structure of the snake AChR binding site, which unlike other muscle AChRs, does not bind α-BTX but does respond to cholinergic ligands. Phosphorylation of AChR, and in particular of its δ-subunit by cAMP dependent protein kinase (PKA), calcium/ phospholipid dependent protein kinase (PKC) and tyrosine-specific protein kinase has been studied. We have mapped the PKA and PKC phosphorylation sites in the δ-subunit and demonstrated that the phosphorylation sites for both enzymes reside in very close proximity, within three consecutive serine residues at positions 360, 361 and 362.

REGULATION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR BY ROTEIN PHOSPHORYLATION, Richard L. Huganir, The Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University New York, New York 10021

The nicotinic acetylcholine receptor is a neurotransmitter-dependent ion channel that mediates the depolarization of the postsynaptic membrane in response to acetylcholine at nicotinic cholinergic synapses. The nicotinic receptor is a M_r-255,000 pentameric complex which consists of four types of subunits α (M_r 40,000), β (M_r 50,000), γ (M_r 60,000) and δ (M_r 65,000) in the stoichiometry of $\alpha_2\beta\gamma\delta$. We have demonstrated that isolated postsynaptic membranes enriched in the nicotinic receptor contain at least four different protein kinases, cAMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase, protein kinase C and a tyrosine-specific protein kinase. Three of these endogenous protein kinases phosphorylate the nicotinic acetylcholine in the isolated postsynaptic membranes. cAMP-dependent protein kinase rapidly and stoichiometrically phosphorylates serine residues on the γ and δ subunits of the receptor. Protein kinase C rapidly and stoichiometrically phosphorylates serine residues on the δ subunit and also causes a slower but significant phosphorylation on serine residues on the α subunit. The endogenous tyrosine kinases which are immunologically related to pp60^{c-src} rapidly and stoichiometrically phosphorylate the β, γ and δ subunits. All of the phosphorylation sites are unique and thus three different protein kinases phosphorylate the acetylcholine receptor on seven different sites.

The phosphorylation sites on the γ and δ subunits for cAMP-dependent protein kinase have recently been identified by protein sequencing techniques. The cAMP-dependent protein kinase phosphorylates serine 353 on the γ subunit and serine 361 on the δ subunit. The locations of the other phosphorylation sites have been proposed to be adjacent to the cAMP-dependent phosphorylation sites' and are located on a common region of each of the subunits with the three phosphorylation sites on the δ subunit being within 20 amino acids of each other. This suggests that phosphorylation of the receptor by all three kinases regulates a common property of the receptor. The phosphorylation sites are located on the major intracellular loop in theoretical models of receptor structure.

Recently we have directly examined the functional effects of phosphorylation of the nicotinic acetylcholine. The ion transport properties of the purified and reconstituted acetylcholine receptor were investigated before and after phosphorylation by cAMP-dependent protein kinase. It was found that phosphorylation of the nicotinic acetylcholine receptor on the γ and δ subunits by cAMP-dependent protein kinase dramatically increased the rate of the rapid desensitization of the receptor, the process by which the receptor is inactivated in the presence of acetylcholine.

Moreover, recent studies have shown that the nicotinic acetylcholine receptor is phosphorylated in intact muscle cells by cAMP-dependent protein kinase and that this phosphorylation regulates the rate of desensitization of the muscle receptor. Primary muscle cells were prelabelled with radioactive inorganic phosphate and then the cells were stimulated with various agents that regulate the intracellular levels of cAMP. Under basal conditions, the receptor was phosphorylated on the β and δ subunits. Addition of forskolin, a potent activator of adenylate cyclase, or cAMP analogues dramatically stimulated the phosphorylation of the δ subunit of the receptor. Exposure of rat primary muscle cells to forskolin and cAMP analogues under identical conditions increases the rate of desensitization of the receptor. The increase in the rate of desensitization after forskolin treatment shows a very similar dose response curve and time course as the increase in the phosphorylation of the δ subunit of the receptor.

These in vivo results together with the in vitro results provide strong evidence that phosphorylation of the nicotinic receptor by cAMP-dependent kinase regulates its rate of desensitization. In contrast, the role of phosphorylation of the receptor by protein kinase C and the tyrosine specific protein kinase is not clear. It has recently been reported that activators of protein kinase C (i.e. phorbol esters) increase the rate of desensitization of the nicotinic acetylcholine receptor in cultured myotubes. This suggests that protein kinase C phosphorylation of the receptor also regulates the desensitization of the receptor. This is not surprising in light of the proposal that the phosphorylation sites on the δ subunit for cAMP-dependent protein kinase and protein kinase C are located within twenty amino acids of each other. Moreover, since tyrosine phosphorylation occurs directly in between these sites on the δ subunit it is likely that desensitization of the receptor is also regulated by tyrosine phosphorylation.

The neurotransmitters or hormones that regulate the activity of these protein kinases and the phosphorylation of the nicotinic acetylcholine receptor have not been identified. However, recent evidence has shown that the neuropeptide calcitonin gene-related peptide (CGRP) is present in the presynaptic terminals at the neuromuscular junction and increases intracellular levels of cAMP in chick myotubes. In addition, CGRP increases the phosphorylation of the nicotinic acetylcholine in primary rat myotubes. CGRP is therefore a likely candidate for a physiological regulator of nicotinic receptor phosphorylation and desensitization.

In conclusion, the nicotinic acetylcholine receptor has provided an excellent model system to study receptor-receptor interactions. It is likely that there are three neurotransmitters or hormones which act on three receptors to regulate the sensitivity of a fourth receptor, namely the nicotinic receptor, through three distinct protein kinase systems.

A STRUCTURAL AND DYNAMIC MODEL FOR THE NICOTINIC ACETYLCHOLINE RECEPTOR

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Folding of the five polypeptide subunits($\alpha_2\beta\gamma\delta$) of the nicotinic acetylcholine receptor (nAChR) into a functional structural model is described. The principles used to arrange the sequences into a structure include: (1) Hydrophobicity → membrane crossing segments (2) amphipathic character → ion-carrying segments (ion channel with single group rotations) (3) molecular shape (elongated, pentagonal cylinder) → folding dimensions of exobilayer portion (4) choice of acetylcholine binding sites → specific folding of exobilayer segments (5) location of reducible disulfides (near agonist binding site) → additional specification of exobilayer arrangement (6) genetic homology → consistency of functional group choices (7) noncompetitive antagonist labeling → arrangement of bilayer helices. The AChR model is divided into 3 parts (a) exobilayer:11 antiparallel β-strands from each subunit (b) bilayer:4 hydrophobic and 1 amphiphilic α-helices from each subunit and (c) cytoplasmic: one (folded) loop from each subunit.

The exobilayer strands can form a closed "flower"(the "resting state") which is opened ("activated") by agonists bound perpendicular to the strands. Rearrangement of the agonists to a strand-parallel position and partial closing of the "flower" leads to a desensitized receptor. The actions of acetylcholine and succinoyl and suberoyl bis-cholines are clarified by the model. The opening and closing of the exobilayer "flower" controls access to the ion channel which is composed of the 5 amphiphilic bilayer helices. A molecular mechanism for ion flow in the channel is given. Openings interrupted by short duration closings (50μsec) depend upon channel group motions. The unusual photolabeling of intrabilayer serines in α, β and δ, but not in γ-subunits near the binding site for non-competitive antagonists (NCAs) is explained along with a mechanism for the action of NCAs such as phencyclidine. The unusual α 192cys-193cys disulfide may have a special peptide arrangement, such as a cis-peptide bond to a following proline. (G.A.Petsko and E.M.Kosower, unpublished results) The position of phosphorylatable sites and proline-rich segments are noted for the cytoplasmic loops.

The dynamic behavior of the AChR channel and many different experimental results can be interpreted in terms of the model. An example is the lowering of ionic conductivity on substitution of bovine for Torpedo δ M2 segment. The model represents a useful construct for the design of experiments on AChR.

TRANSITIONS IN MOLECULAR FORMS OF ACETYLCHOLINESTERASE DURING THE DEVELOPMENT OF DROSOPHILA

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The major molecular form of native acetylcholines terase (AChE, EC 3117) present in the heads of adult Drosophila is an amphiphilic dimer (G2) associated to the membrane through a glycolipid anchor (references a,b,c). We have investigated the AChE molecular forms present in late embryos (E), larval stages L1,L2 and L3, and pupae (P). Entire animals were homogenized in a low salt buffer containing 1% Triton X100 and a cocktail of anti proteolytic agents. These extracts were analyzed by ultra centrifugation in sucrose gradients in the presence of Triton X100, Brij 96 or in the absence of detergent and by non denaturing electrophoresis.

The AChE activity expressed as nmoles AcSCh hydro lyzed/min/g of wet weight showed a transient peak at the L1 stage, reincreased in pupae and was maximal in the adult.

Figure 1 shows that four molecular forms (at most) were present at every developmental stage (bands 1, 1', 2 and 3). By extensive correlation between AChE peaks in sucrose gradients with these bands on non denatu ring electrophoresis and by analogy with the adult enzyme, we demonstrated that the four electromorphs corresponded to an amphiphilic dimer (1), an amphiphilic monomer (1') and hydrophilic dimer (2) and monomer (3).

Since endogenous proteolytic activity during metamorphosis of Drosophila is high (see ref. d), we checked that the molecular forms observed in this study did not result from the breakdown of the major G2 form
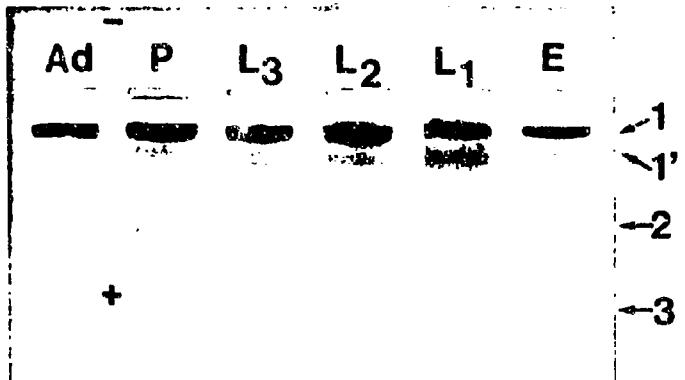
Figure 1:Non denaturing electrophoresis of AChE present in crude extracts of late embryos (E), larval (L), pupal (P) and adult (Ad) Drosophila. The gel (7.5% acrylamide) and the running buffer contained 0.5% Triton X100. Migration for 3 Hours at 10 V/cm.

during the homogenization step.

Our data support the hypothesis that a native amphiphilic G_1 form of AChE occurs during the development of Drosophila. The existence of this native form at the L_1 and pupal stages (when AChE is actively synthesized) further suggests that amphiphilic G_1 form might represent the precursor of the amphiphilic G_2 form which is the mature, membrane-bound form of the enzyme.

Developing Drosophila (particularly L_1 and P stages) therefore constitutes a favourable system for the study of the biosynthesis of the glycolipid-anchored molecules of AChE. For example it should be possible to demonstrate, with this model, whether the amphiphilic monomer possesses a glycolipidic domain or a hydrophobic sequence which could be further exchanged (see ref. e).

References: a)JP Toutant, M Arpagaus, D Fournier. J Neuro chem, in press. b) D Fournier, J Bergé, C Bordier.J Neuro chem, in press. c) AL Gnagey, M Forte, TL Rosenberry (87) J Biol Chem, in press. d) SA Dewhurst, RE McCaman WD Kaplan (70) Biochem Gen 4,499-508. e) Cross GAM (87) Cell 48,179-181.

POLYMORPHISM OF MYOSIN LIGHT CHAINS AND ACETYLCHOLINESTERASE DURING THE DEVELOPMENT OF NEW-BORN RABBIT FAST AND SLOW DENERVATED MUSCLES

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The rabbit Semimembranosus proprius (SMp) and Semimembranosus accessorius (SMa) muscles represent a good model for studying the transformations of muscle properties during the postnatal differentiation. In the adult, these muscles are respectively homogeneous in slow twitch (SMp) and fast twitch (SMa) fibers[1]. However, they are heterogeneous at birth, and express their adult characteristics from two months postnatal onwards. During this period, we have studied the influence of motor innervation on the development of their properties, particularly at the level of acetylcholinesterase (AChE) molecular forms and myosin slow (LCs) and fast (LCf) light chains.

The postnatal alteration of SMa and SMp muscles is characterized by the disappearance of the neonatal heterogeneity and the acquisition of the homogeneous fast or slow fiber type pattern. The fibers of these muscles denervated at birth are altered differently : dramatic atrophy of fast twitch fibers whatever muscles are studied, preservation of SMp slow twitch fibers characteristics and fatty degeneration of SMa.

At birth, both muscles present a similar pattern of myosin fast and slow LC. In control muscles, the alteration of fiber populations to homogeneous types lead to the disappearance of supernumerary chains from 15 days onwards. In the slow muscle, neonatal denervation prevents LCf disappearance. In the fast muscle, denervation influences essentially the installation of LCf which is delayed by 15 days.

At birth, the polymorphism of AChE is similar in SMp and SMa muscles. One month after denervation, the specific activity of AChE is twice that of control. Its polymorphism is not much perturbed, while in the adult denervation induces an important increase in AChE specific activity (x10) and particularly a great alteration in its polymorphism according to the fast or slow muscle fiber types[2,3].

REFERENCES.

1. Appel H.J. and Hammersen F. 1979. Cell Tissue Res., 196, 531.
2. Bacou et al. 1982.Nature, 295, 661.
3. Lai et al. 198 . Nature, 321, 72.

ACETYLCHOLINE RELEASE FROM RAT BRAIN SYNAPTOSOMES AS MEASURED BY
THE CHEMILUMINESCENCE METHOD

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Rat brain synaptosomes (RBS) were prepared by density-
gradient ultracentrifugation on an isotonic Ficoll density
interface at ph 7.4 and 4° C. Release of acetylcholine (ACh) was
measured by the choline oxidase-peroxidase-luminol method at pH
7.8-8.2. RBS were polarized with KCl, gramicidin and the
calcium ionophore   37. The amount of ACh released was 1% of
that released by T edo synaptosomes of the same protein
content. Release induced by KCl is calcium dependent and follows
an identical time course to the flunarizine-independent increase
in intracellular free Ca which has been observed in fluorescence
measurements with Indo-1. The prompt ACh release is accompanied
by a slow (34 min t.c.) calcium-independent release of choline,
a. identified by measurements performed after 30-60 min of
incubation with 10 micromolar phospholine. The synaptosomes
release an interfering substance when lysed with detergent. Total
ACh content was measured after trichloroacetic acid extraction
and oxidation with sodium metaperiodate. Substantial amounts of
choline are present in the initial preparation. After the
synaptosomes have been stored for 32 hr, ACh release is still 30-
50% of initial values.
     Ceteidil blocks the release of ACh from RBS depolarized with
KCl and gramicidin as it does in Torpedo synaptosomes. The
apparent Ki for ceteidil is approximately 25 micromolar. There
is no effect of ceteidil on choline release.

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NEGATIVELY CHARGED PHOSPHOLIPIDS, A POSSIBLE TARGET FOR THE PRESYNAPTIC PHOSPHOLIPASE NEUROTOXIN FROM SNAKE VENOMS

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Crotoxin, the major toxic component of the South American Rattlesnake, **Crotalus durissus terrificus**, is a potent neurotoxin which possesses a phospholipase A_2 activity and blocks neuromuscular transmission primarily at the presynaptic level, although at higher doses it also reduces the postsynaptic response to acetylcholine by stabilizing the cholinergic receptor in an inactive conformational state.

Crotoxin, which is in fact a mixture of very similar isoforms, consists of two non identical subunits. The basic component-B carries the phospholipase A_2 activity of the toxin and possesses a low toxicity and the acidic component-A has no enzymatic activity although it resembles a phospholipase A_2 in its primary structure. Component-A, is not toxic by itself but considerably enhances the lethal potency of the phospholipase component-B. Upon interaction with biological or artificial membranes, the two subunits dissociate: component-A is released free in solution and component-B is bound. The isolated phospholipase component-B binds in a non saturable manner to either erythrocyte or postsynaptic membranes. Component-A which does not bind to membranes, considerably reduces the non specific adsorption of the phospholipase subunit, without preventing its saturable (specific) binding to a limited number of binding sites on the synaptic membrane.

The isolated component- possesses a low affinity for unilamellar vesicles constituted of zwitterionic phospholipids, but binds with a high affinity to negatively charged phospholipids. The non enzymatic component-A enhances the selectivity of component-B for negatively charged phospholipids since it completely inhibits the low affinity binding of component-B to vesicles of zwitterionic phospholipids. These observations strongly suggest that negatively charged phospholipids are the physiological target of crotoxin or at least an important part of this target. This hypothesis implies that, at variance with other plasma membranes, the presynaptic plasma membrane (or some specialized area of the plasma membrane) exposes negatively charged phospholipids on its external surface.

VERATRIDINE-INDUCED ASYNCHRONOUS QUANTAL RELEASE OF TRANS-MITTER IN EXCITATORY AND INHIBITORY NERVE-MUSCLE SYNAPSES OF CRAYFISH

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10^{-5}-10^{-4} mol/l veratridine in the superfusate caused within seconds high-frequency asynchronous release of excitatory and inhibitory transmitter quanta in crayfish neuromuscular junctions. This quantal release gave rise to vigorous current fluctuations in the postsynaptic membrane of voltage clamped muscle fibres. The current fluctuations were evaluated by means of noise analysis and the total amount of transmitter quanta liberated in the reaction with veratridine was estimated. After veratridine was applied, in many fibres of the claw opener muscle the quantal release rate $\tilde{n}$ increased within seconds from $\tilde{n} < 1$ quantum/s to a maximum $\tilde{n}(max) \approx 5,000$-15,000 quanta/s. Thereafter, $\tilde{n}$ declined exponentially either with a single or with two time constants. For excitatory quantal release the single time constant for the exponential decay of $\tilde{n}$ was $\tau \approx 55$ s while it was $\tau \approx 75$ s for inhibitory quantal release. Altogether, in a single reaction with veratridine at the claw opener muscle, the average total number of excitatory quanta released from the terminals on a fibre of ≈ 1 mm length was $\tilde{s} \approx 300,000$. The equivalent number of inhibitory quanta was $\tilde{s} \approx 600,000$. Veratridine could induce vigorous quantal release only once in a single muscle fibre suggesting exhaustion of quantal stores of transmitter by veratridine. The size, $\tilde{s}$, and the decline, τ, of the readily releasable quantal store of transmitter obtained by veratridine are similar to results obtained by 100 mmol/l K^+ (Martin and Finger 1985, Neurosci Lett 53:309-314). Stimulation at 40 Hz of the excitatory nerve for >40 min caused release of >5 million quanta, and at this time

there was still no sign of transmitter deficiency. Possibly, transmitter recycling or synthesis occurs during repetitive nerve activity. Such processes might be absent, however, during strong tonic depolarizations of nerve endings (Finger and Martin 1987, Pflügers Arch 408 ,Suppl 1,:R67/260).

In the abdominal superficial extensor muscle in which fibres are innervated by two excitatory axons up to $\tilde{a} \approx$ 3,000,000 excitatory quanta could be released from the terminals on a 2-mm-long fibre by application of veratridine.

In low-Ca^{++} superfusate (normal Ca^{++} removed), about 75 % of the total number of releasable excitatory quanta could be liberated by veratridine in the claw opener. In the abdominal superficial extensor muscle only 8 % of excitatory quanta could be released in low-Ca^{++} saline. This suggests a different dependence of quantal release on extracellular Ca^{++} for these two nerve-muscle preparations (Finger and Martin 1987, Neurosci Lett 75:293-298). When extracellular Na^+ was replaced by Li^+, the total amount of excitatory and inhibitory quanta liberated by veratridine was 70-90 % lower than that obtained in normal superfusate. The remaining quanta were released, however, after normal Na^+ was reestablished and Li^+ removed from the bath.

MOLECULAR CLONING AND STRUCTURAL CHARACTERIZATION OF HUMAN CHOLINESTERASE GENES

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Cholinesterases (ChEs) are highly polymorphic proteins capable of rapidly degrading the neurotransmitter acetylcholine. ChEs can be differentiated by their catalytic properties into two major classes, both expressed in various tissues. These are acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), which exist in several molecular forms, composed of a single, two or four catalytic subunits. Heavier forms contain collagen-like "tail" non-catalytic subunits, covalently bound to one up to three tetramers as above [1]. It is not known as yet whether different genes code for the various ChE forms, or whether control is post-transcriptional.

ChEs in various species display extensive homology at the level of their amino acid sequence [2,3] and appear to be derived from a single ancient gene [4]. Homology was retained also at the level of nucleotide sequence and between evolutionarily-remote species such as Torpedo californica and man [5]. In humans, the genes coding for ChE are intensively expressed in various embryonic tissues, including oocytes, as was shown by in situ hybridization to frozen ovarian sections [6]. This makes the ChE genes good candidates for the appearance of inheritable, re-inserted processed genes. In addition, genetic linkage studies have shown that defects in the expression of serum ChE may be linked to either chromosome 3 genes like transferrin [7] or chromosome 16 genes such as haptoglobin [8]. Indeed, chromosomal mapping of human ChE genes by in situ hybridization revealed three loci containing ChE-coding sequences, two on chromosome No. 3 and one on chromosome No. 16 [9].

In order to find out how many functional ChE genes exist in humans, what their structure and mode of regulation are and which of the polymorphic ChE forms they encode, molecular cloning and gene isolation experiments were initiated. Several different phage libraries of human genomic DNA fragments were screened, including complete genomic libraries, libraries enriched by size fractionation of enzymatically restricted DNA and libraries prepared from isolated chromosomes. The full-length cDNA clone coding for human ChE³ or fragments isolated from this clone [5] were labeled and used as probes in these experiments. The screening resulted in the isolation of several different DNA fragments, which were subjected to DNA sequencing by the Sanger technique, using M13 single stranded phages and synthetic oligodeoxynucleotide primers synthesized according to the ChEcDNA sequence.

The existence of differently restricted fragments from the ChE genes on two different chromosomes, together with the results obtained by in situ hybridization to chromosomes and DNA blot hybridization studies, indicate the presence of at least three ChE genes in humans. One of these, localized on chromosome No. 3, appears to contain at least three intervening sequences and may represent the original human ChE gene. Two other ChE genes appear to be processed genes derived from the latter by post-transcriptional processing events. These do not contain introns within their coding regions and they include point changes in nucleotides as compared with the human ChEcDNA. The two processed ChE minigenes are localized on chromosomes 3 and 16, respectively, and the ChE minigene on chromosome 16 is most probably an actively expressed processed gene, coding for the C5 variant of serum ChE [8].

In order to define the structural organization and intrinsic differences between the various human ChE genes, pulse-field DNA electrophoresis followed by DNA blot hybridization is currently employed, aiming to identify, clone and isolate the human ChE genes in their fully intact forms so that their promoter regions and expression properties can be approached.

References
1. Massoulie, J. and Bo, S. (1982) Ann. Rev. Neurosci. 5, 57-106.
2. Schumacher, M., Camp, S., Manlet, Y., Newton, M., McPhee-Quigley, K., Taylor, S. S., Friedman, T. and Taylor, P. (1986) Nature 319, 407-409.
3. Prody, C., Zevin-Sonkin, D., Gnatt, A., Goldberg, O. and Soreq, H. (1987) PNAS 84, 3555-3559.
4. Mori, N., Itoh, N. and Salvaterra, P. M. (1987) PNAS 84, 2813-2817.
5. Soreq, H. and Gnatt, A. (1987) Molecular Neurobiology, in press.
6. Zakut, H., Malinger, G. and Soreq, H. (1987). This conference.
7. Sparkes, R. S., Field, L. L., Sparkes, M. C., Crist, M., Spence, M. A.., James, K. and Garry, P. J. (1984) Hum. Hered. 34, 96-100.
8. Lovrien, E. W., Magenis, R. E., Rivas, M. L., Lamvik, N., Rowe, S., Wood, J. and Hemmerling, S. (1978) Cytogenet. Cell Genet. 22, 324-326.
9. Zamir, R., Sindel, L., Zakut, H. and Soreq, H. (1987). This conference.
10. Maniatis, T., Fritsch, E. F. and Sambrook, J. Cold Spring Harbor, 1982.
11. American Type Culture Collection.

ENERGETICS OF ACTIVATION OF THE ACETYLCHOLINE RECEPTOR. Meyer B.
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Mathematical techniques applicable to a system with an
arbitrary number of channels (Jackson, 1985, Biophys. J. 47:129)
were used to analyze the channel gating kinetics of the
acetylcholine receptor from 0 to 20 µM carbachol. This analysis,
which included fitting single channel dose-response curves to
appropriate theories, provided estimates of the unliganded,
singly liganded, and doubly liganded receptor channel gating rate
constants and equilibrium constants. The dissociation constants
to the first and second binding sites were estimated for a closed
channel, and the principle of detailed balance was used to
calculate the dissociation constants to the receptor with an open
channel. This constitutes a complete energetic description of
this allosteric protein.

The equilibrium constants of the gating transitions are
1.9×10^{-6}, 4.4×10^{-4}, and 14 for unliganded, singly liganded,
and doubly liganded receptor, respectively. For a closed
channel, the first dissociation constant is 5 µM and the second
is 10 mM. For an open channel, the two dissociation constants
are .025 and .31 µM, respectively. Thus the binding strengths of
the two sites are very different when the channel is closed but
very similar when the channel is open. The binding sites for the
closed state differ further in the degree to which binding
accelerates the rate of opening. Binding at the first site
accelerates the rate of opening 40 fold over the rate of
spontaneous opening; binding at the second site accelerates the
rate of opening an additional 7000 fold.

These results can be interpreted by considering how
binding energy can be utilized by the receptor. For the first
binding site much of the energy of binding is utilized to
stabilize the complex with the closed state. The binding of the
second ligand to the closed receptor channel is weaker because
more of the binding energy is used to stabilize the open channel
conformation.

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The control of synaptic functions by membrane proteins has seduced even the most critical
scientists to consider the central factor to be a local molecular mechanism. However,
even the details of the amino acid sequences have taught nothing about the physical basis
of membrane excitation. Membrane theory, in contrast, still continues to proceed along
unrelated and mutually exclusive lines:
- chemiosmotic theories, where protons are crucial, describe the observed free energy
  coupling;
- electrical theories, where protons are ignored, model mathematically the deterministic
  shape of propagating action potentials;
- allosteric theories, where neither protonic free energy nor deterministic propagation
  matter,were to explain the fluctuating ion channels.
In contradiction to any molecular interpretation, though, the observed membrane excitation
is m a c r o s c o p i c . Action potentials, for example, extend for some millimeters
in space in the squid giant axon membrane. Ion channels obey macroscopic statistical fluc-
tuations. Protonic free energy coupling, too, since reversible, requires a cooperative
property of a macroscopic number of membrane molecules.

This situation is reminiscent of the propagation mechanism of sound which would have
remained unknown were it investigated on the level of single, isolated molecules.

The macroscopic action potential likewise obeys the principles of thermodynamics and can
therefore be neither molecular nor purely electrical in nature. The thermodynamic proper-
ties of the macroscopic membrane surface itself are not considered in the above membrane
theories. This surface is constituted from the hydrated phospholipid layers and does not
require the presence of proteins a p r i o r i . The application of classical thermo-
dynamics gives rise to remarkable responses of phospholipid layers to stimulation, e.g.,
by ATP or ACh hydrolases:
- free energy-rich proton-phospholipid bilayer conformations consistent with local and
  with global aspects of chemiosmotic coupling;
- propagation of the hydrodynamic excitation along the bilayer with saltatory velocity
  of sound and accompanied by increase and decrease of temperature, membrane displacement,
  electrical polarisation, and optical density in consistence with the observed action
  potential;
- fluctuating opening and closing of aqueous ion channels across the otherwise impermeable
  lipid bilayer lattice due to the strength of the thermal motion of the flexible surface
  in the more compressible states.

A proof for the lipid bilayer mechanism of biological membrane functions can be obtained
in any one of the following ways:

- Urification of the previously unrelated theories of chemiosmosis, action potential,
  and ion channel fluctuations by the entropy, forces, and fluctuations in the lipid
  bimolecular layer.
- Evidence of the mechanism which is free of adjustable parameters and only requires
  existence of the bilayer, validity of classical thermodynamics, and applicability
  of Langmuir monolayer diagrams of state.
- Resolution of long-standing paradoxes:
  Hill's observation of temperature increase and d e c r e a s e during the excitation;
  Tasaki's observations of mechanical and optical pulses during the action potential,
  and its persistence in the absence of monovalent cations;
  the presence of ion channel opening and closing in the absence of proteins;
  the dependence even of protein-induced ion channel fluctuations on the lipid surface
  variables of pressure, electrical potential, or pH.
  Crucial experimental test of the theory:
  the predictable induction of ion channels in pure phospholipid bilayers by the electro-
  chemical proton potential, by surface pressure, or by temperature, as observed by
  Hanke, Silman, Corcia, and myself;
  the appearance of deterministic millisecond pulses comparable to action potentials
  in pure lipid bilayers, induced by reversible pulses in any one of the surface variables;
  free energy-coupling conformational bilayer transitions induced by protons directly or
  by ATP and ACh hydrolases.

The results of experiments on pure lipid bilayers in collaboration with Israel Silman,
Wolfgang Hanke, and Avus Corcia, as well as of the hydrodynamic theory for the electro-
mechanical coupling during action potentials in collaboration with Helmut Brand, are
striking, since no other membrane component is capable of explaining ion channels and
excitation but the macroscopic phospholipid surface. These are and will be reported elsewhere.

In consequence, sensory receptors couple free energy into the phospholipid entropy,
propagation of the excitation is by the mechanism and with the velocity of first sound,
while ion channels open and close, controlled by the strength of the thermal motion.
The sensory stimuli, whethe peripheral or synaptic, mediated by proteins or directly
applied, induce the bilayer excitation via any one of the phospholipid surface variables:
electrical or chemical potential of the protons; mechanical displacement; electrical
polarisation; electrochemical potential of calcium and other aqueous ions or hydrophobic
membrane constituents. Receptors for voltage, pH, acoustical sound, for photons, taste
and smell seem predictable, respectively.

Synaptic transmission is reversible if the junction is tight, in the order of the mechanical
displacement; this is some 10 Å according to Iwasa and Tasaki. Transmission is also con-
trolled by the chemiosmotic proton potential. The cholinergic synapse is thus controlled
by the fastest protonic free energy source known in the nervous system and might therefore
be even more efficient than the mitochondrial ATPase system: the catalytic activity of the
phospholipid-anchored membrane acetylcholinesterase. Thus, free energy coupling across
synapses is controlled in a chemically modifiable fashion.

ACh
↓
$H^+ \rightarrow H^\circ$

receptor   lipid membrane   excitation   synapse
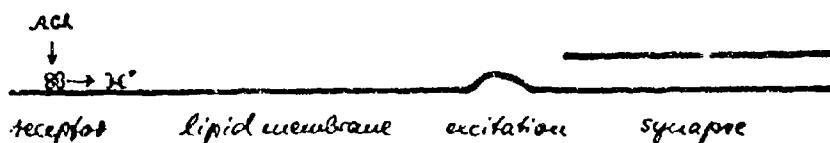
SPONTANEOUS RELEASE OF ACETYLCHOLINE FROM THE TORPEDO ELECTRIC
ORGAN

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In the present work we investigated the mechanisms
underlying spontaneous acetylcholine (ACh) release. This was
done by characterizing the release of ACh from Torpedo electric
organ neurons and the effects thereon of cholinergic ligands.

Incubation of Torpedo electric organ prisms in modified
Torpedo buffer in the presence of the acetylcholinesterase (AChE)
inhibitors phospholine iodide (100 uM) or paraoxon (150 uM)
results in spontaneous ACh release. Release is linear for about
10 min after which it levels off to a plateau. The inhibition of
ACh release at the plateau is completely lost when electric organ
prisms are transferred to fresh media, whereas when the tissue is
transferred to medium which has been preincubated with electric
organ prisms, release is completely blocked.Inhibition of ACh
release by the conditioned medium is abolished by heating the
media to 90°C for 10 min. Incubation of Torpedo electric organ
prisms in the absence of AChE inhibitors results in basal ACh
release whose initial rate (0-10 min) is similar to that observed
in the presence of AChE inhibitors. However, at longer
incubation times (10 - 30 min) ACh release in the absence of AChE
inhibitors is not inhibited and proceeds at the initial rate.

The effects of the vesicular ACh uptake inhibitor 2(4-
phenylpiperidino) cyclohexanol (AH5183) on basal ACh release were
examined. Addition of AH5183 (0.1-1 uM) in the absence of AChE
inhibitors results in repressed basal release whereas in the
presence of either phospholine iodide or paraoxon AH5183 does not
affect ACh release. Spontaneous ACh release and the effects
thereon of AH5183 are pH dependent. Maximal release is obtained
at acidic pH whereas release is most sensitive to AH5183 at
neutral and basic pH.

PRESSURE EFFECTS ON THE CARBAMYLATION OF BUTYRYLCHOLINESTERASE

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The effect of hydrostatic pressure on the reaction of human plasma
cholinesterase (EC.3.1.1.8) tetrameric form with the carbamyl ester, N-
methyl- (7-dimethylcarbamoxy) quinolinium iodide, was studied under single-
turnover conditions at 35°C up to 0.8 kbar.

The enzyme carbamylation was followed by monitoring the formation of
the fluorescent quinolinium ion in a high-pressure stopped-flow apparatus
operating in fluorescence mode. The apparent activation volume change (ΔV^+)
accompanying the reaction was determined from the pressure-dependence of the
carbamylation rate. ΔV^+ consists of two contributions : the volume change of
the substrate binding step (ΔV_b) and the activation volume change of the
carbamylation step (ΔV_c^+). These two contributions were obtained from
experiments performed under pressure at different non-saturating substrate
concentrations.

The rate of carbamylation displays a biphasic non-linear pressure
dependence. Up to 0.4 kbar, the non-linearity is caused by a change in the
enzyme-substrate dissociation constant (K_D) and in the carbamylation rate
constant (k_2) with pressure. The curvilinear plots (fig. 1) are the fit of
the experimental points according to the equation :

$$k_{obs} = \frac{k_2 \exp (-p \, \Delta V^+_{k_2} \, / \, RT)[S]}{K_D \exp (-p \, \Delta V K_D \, / \, RT) + [S]}$$

with (fig. 2)

$$\Delta V^+ = -RT \left(\frac{\partial \ln k_{obs}}{\partial p} \right)_T = \Delta V^+_{k_2} - \frac{1}{1 + S/K_D} \cdot \Delta V_b$$

From the pressure-induced changes in K_D and k_2, we calculated
$\Delta V_b = -129 \pm 15 \text{ ml.mol}^{-1}$ and $\Delta V^+_c = 119 \pm 10 \text{ ml.mol}^{-1}$ for the reaction at
atmospheric pressure. These large values cannot be simply explained in terms
of molecular interactions ; they could reflect important structural changes.

Therefore, the volume change associated with each step must be regarded in the sum of individual contributions : bonds formation/disruption, conformational changes and water structuration changes.

Beyond 0.4 kbar, the curvatures become more marked and the inhibition of the carbamylation occurs in a narrow pressure range. The corresponding great positive ΔV^+ suggest that, above 0.4 kbar, pressure induces a conformational change and/or an extensive hydration change of the enzyme. However, high-pressure electrophoresis data show no change in quaternary structure and no unfolding of the enzyme. In addition, the inhibition is reversible after pressure release.

At low substrate concentration (S = 10 µM), the pressure effects are markedly different. Since ΔV^+ is dominated by the binding contribution, the carbamylation is not pressure-inhibited. Surprisingly, ΔV^+ appears to be pressure-independent at least up to 0.8 kbar. Data can be fitted by a straight line assuming $\Delta V_b = -5$ ml.mol^{-1} and $\Delta V_o^+ = o$.

The pressure effect depending on substrate concentration could be discussed in terms of enzyme conformation perturbation. It may be suggested that the binding of substrate induces a conformational change leading to a pressure-sensitive conformational state.
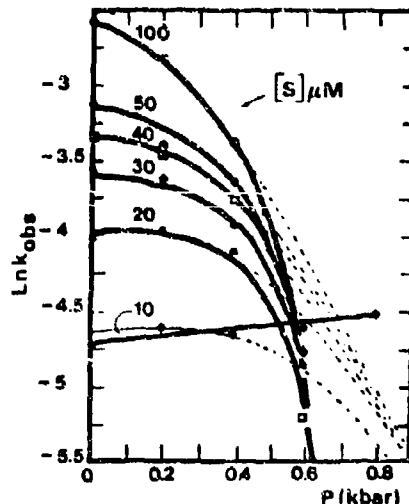

Fig.1 — Pressure dependence of the observed rate c carbamylation at 35°C, pH = 7.4 ; e = 6.25 IU.µl^{-1} (1.U: µmol BuSCh hydrolysed.min^{-1} at Vmax in 0.1M phosphate pH 7, 25°C).
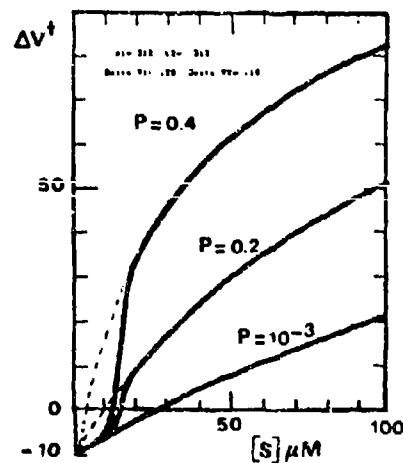
Fig.2 — Apparent activation volume change (ΔV^+) as a function of carbamyl ester concentration (µM).

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A NEUTRALIZING MONOCLONAL ANTIBODY TO CROTOXIN

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The purpose of this study was to define the immunological relationships among rattlesnake neurotoxins by using monoclonal antibodies. Crotoxin is a potent presynaptic neurotoxin found in the venom of the South American rattlesnake, *Crotalus durissus terrificus*. The toxin is a heterodimeric protein composed of an acidic and a basic subunit. Mice were immunized with either the intact toxin or its basic subunit. Monoclonal antibody-secreting hybridoma cell lines were then obtained using standard techniques. One of the lines was found to produce antibody (Ab 1) which neutralized crotoxin and became the subject of further study.

After production from ascites tumors, Ab 1 was purified on a protein A-Sepharose column and typed as subclass IgG_1. Ab 1 reacted with crotoxin and the related mojave and virgrandis toxins, but did not react with concolor toxin or phospholipase A_2s from *C. atrox* or *C. adamanteus*. Mouse lethality of both purified crotoxin and mojave toxin was neutralized by Ab 1. Ab 1 was approximately 50-fold more effective at neutralizing purified crotoxin than was commercial horse antiserum. However, Ab 1 was ineffective at protecting from lethality due to *C. d. terrificus* venom under conditions where the horse antisera worked well. Paradoxically, Ab 1, when added to horse antisera, markedly potentiated the latter's effectiveness at protecting from *C. d. terrificus* venom.

We conclude that several, but not all rattlesnake neurotoxins have a common epitope and that antibodies against this epitope can neutralize the toxin, but not necessarily the venom.

THE EFFECT OF AH 5183 ON THE RELEASE AND STORAGE OF NEWLY SYNTHESIZED ACETYLCHOLINE IN FROG MUSCLE

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It has been reported that the compound AH 5183 is a potent inhibitor of acetylcholine (ACh) uptake in vitro into synaptic vesicles prepared from the electric organ of the electric ray (see for review: ref. 1). In the present experiments we present evidence strongly suggesting that AH 5183 has a selective inhibitory effect on ACh uptake into synaptic vesicles of preparations of frog skeletal muscle.

Sartorius muscles of R. escul ta were treated with an irreversible cholinesterase inhibitor (soman) and then stimulated via the nerve for 30 min (1 per s trains, 100 Hz for 0.1 s). Muscles were left in Ringer containing 50 μM choline-d9 for 3 h and then stimulated for a second 30 min period. AH 5183 (10 μM) was or was not present throughout the incubation. ACh-do and ACh-d9 were measured by mass spectrometry in incubation medium, 'bound', presumably vesicular, and 'free' extracts of the tissue (2).

It was found that ACh release was exhausted by 30 min stimulation and that a 3 h rest period was sufficient to restore the pools of both releasable and bound ACh. Whereas AH 5183 had no effect on ACh released during the first period it caused an about ten-fold decrease of the newly synthesized ACh-d9 released during the second period. Further, AH 5183 strongly reduced the ACh-d9 which was incorporated into the bound fraction. AH 5183 had no inhibitory effect on the amounts of ACh-d9 synthesized during the period of rest after the first stimulation period.

It is concluded that, whereas AH 5183 did not influence the depletion of the releasable ACh pool per se, it had a strong inhibitory action on both the regeneration of releasable ACh and the transfer of ACh from the free into the bound ACh fraction of the muscle.

Parsons S.M. et al. (1987) Ann.N.Y.Acad.Sci. 493, 220-233.
Miledi R. et al. (1982) J.Physiol. 333, 189-199.

AN ANTISERUM SPECIFIC FOR THE MEDIATOPHORE, AN ACETYLCHOLINE RELEASING

PROTEIN, EXHIBITS A PRESYNAPTIC BINDING IN TORPEDO AND RAT

NEUROMUSCULAR JUNCTIONS.

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Recently we purified from the presynaptic plasma membrane of
Torpedo electric organ a protein (the mediatophore) that mediates
calcium dependent release of acetylcholine from proteoliposomes
(Israel et al, 1986, Proc.Natl.Acad.Sci.USA, 83, 9226-9230).
By immunoblot techniques, using a rabbit antiserum to the purified
mediatophore, this antigen was shown to be most abundant in presynaptic
plasma membranes purified from Torpedo electric organ, present in lesser
amounts in synaptic vesicle fractions and hardly detectable in electric
nerve membranes or plasma membranes of the electroplaques. It was also
possible to demonstrate by indirect immunofluorescence the presence of
a related antigen in Torpedo and rat neuromuscular junctions. The
fluorescence pattern is typical of a presynaptic binding. These
immunological results show that the protein mediatophore is specific for
the presynaptic plasma membrane and that a related protein is also present
in Torpedo and rat neuromuscular junctions.

INHIBITORY EFFECT OF CETIEDIL ON PRESYNAPTIC ACETYLCHOLINE
RELEASE

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Manaranche R.

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Cellulaire et Moléculaire, C.N.R.S., Gif-sur-Yvette, France.

The effects of cetiedil, a vasodilatator substance, on
cholinergic function, were examined at the nerve electroplaque
junction of Torpedo marmorata using both synaptosomes and
slices of intact tissue. Cetiedil abolished the calcium
dependent release of acetylcholine (ACh) triggered by
depolarization with KCl, gramicidin D application, Glycera
neurotoxin effect or by the addition of the calcium ionophore
A23187, placing the site of action after the calcium entry step.
In addition, a direct effect on the release process itself was
supported by the fact that cetiedil was able to block the calcium
dependent release of ACh mediated from ACh-containing
proteoliposomes reconstituted from synthetic lecithin and the
recently isolated presynaptic membrane protein "mediatophore".
This effect was obtained at a Ki close to that observed in
synaptosomes or in intact tissue (5-8 micromolar). Under the
conditions used in these experiments, neither the synthesis,
nor its compartmentation within the nerve terminal were altered.
However, the drug appears able to reduce high-affinity choline
uptake and the vesicular ACh incorporation when given together
with radioactive acetate precursor. Thus, cetiedil has a broad
inhibitory action on cholinergic membrane uptake process.

REGULATION OF CHICKEN MUSCLE ACETYLCHOLINE RECEPTOR GENE EXPRESSION

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The nicotinic acetylcholine receptor (nAChR) within the electric organ and vertebrate skeletal muscle has been shown to be an oligomeric, integral membrane, ion-gating, glycoprotein consisting of four subunits of a stoichiometry $\alpha_2\beta\gamma\delta$. The analysis of complementary DNA (cDNA) and genomic equivalents has shown than within the electric organ each polypeptide is encoded by a distinct gene. Parallel studies on the calf nAChR reveal a similar pattern of genomic organisation. Regulation of the expression of these genes in vivo is of great interest but as yet little information is available on this matter. We have taken advantage of the accessibility of the chicken to investigate the developmental expression of the chicken skeletal muscle nAChR transcripts, and their response to chronic denervation. Subunit-specific probes derived from cDNA and genomic clones generated within this laboratory for the α,β,γ and δ subunits of the chicken muscle nAChR have been used to probe poly(A)$^+$ RNA derived from embryonic, denervated and innervated chicken skeletal muscle. High levels of each subunit transcript are seen early in development peaking at D_{12}-D_{14}, decreasing rapidly towards D_{18}. Much lower levels of the α,β and δ transcripts could be detected in innervated muscle. Upon denervation of 8-week-old chicken muscle, levels of all four transcripts were observed to approach embryonic values. The importance of these results will be discussed with regard to the regulation of the genes encoding the nAChR oligomer.

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regulation of the genes encoding the nAChR oligomer.

HUMAN MEMORY MODELLING : SYNAPSE LEVEL

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The stable synapse-modification (a base of the long-term space traces) is connected with the molecular-chemical processes in the synapse forms, the genetic apparatus of the neurons taking part. Two basic hypotheses dominate :

- The adaptation hypothesis - a sequence of the following events :
1. Increasing of the secretorius functions of the synapse when it is included in the activated system of inter-neuron links, which lead to intensification of the interaction between the mediator and the receptor proteins of the post-synapse membrane; 2. Causing of increased forming of the depressor (in the synthesis of the receptor protein in the membrane) and its diffusion in the nucleus in an increased quantity; 3. The synthesis of the corresponding mRNA in the nucleus is increased and also its coming out in the cytoplasm, which to increasing of the receptor protein synthesis; 4. With the help of the factor "building in"(which is formed in the membrane when the synapse is activated) increased building in of accessory ammounts of this protein in the sub-synapse membrane is caused and as a final result the synapse effectiveness increases. Consequently the basic plastic processes in the synapse apparatus take place in the post-synapse membrane and probably the apparatus of the spine growths takes part in the memory processes and the teaching. Despite the existence of a lot of indirect facts, there is not a direct provement of the adaptation hypothesis up to now because of the absence of an adequate biological model ;

- A structure-chemical hypothesis : The role of the genome is reduced to securing of the synthesis of specific (for determined neurons) proteins or peptids, i.e. to securing of markers, which determine the genetic type of the brain structures (one for all) and to forming of the inborn (unconditional) reflexes. The genetically determined neuron systems (proto-chains) are in fact a structural brain code with high plasticity, i.e. the record and storing of the ontogenetic information in the Long-term Memory (LM) is realized in a chemical way. The plasticity features secure forming of new neuron chains (meta-chains), i.e. synapse-modifications are realized (the increase of the synapse effectiveness is determined anatomically; new synapse networks are formed). Of most complete character is the hypothesis of G. Ungar, namely : 1. The simultaneous activation of the neuron chains (of the unconditional reflex) and some of the afferent systems leads to a trans-synapse transport of the peptids-markers in the network of specialized inter-neurons ("memory cells"); 2. The trans-peptidases of the last cells "sew together" from the markers a new peptide - a non-genetic level of coding of the ontogenetic information; 3. By building in of the "sewn together" peptide in the membrane synapse protein (of the "memory cells") the links of the newly-formed meta-chains are consolidated, i.e. a conditioned reflex link appears.

IN SEARCH OF THE LIGAND BINDING SITE OF THE SNAKE NICOTINIC ACETYLCHOLINE RECEPTOR

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The acetylcholine receptor (AChR) in the neuromuscular junction (NMJ) of Elapid snakes responds to cholinergic ligands but unlike other muscle AChRs, does not bind α-bungarotoxin (α-BTX). Hence, dissecting the structure of the snake AChR and its cholinergic binding site is of unique interest. By determining the amino acid sequence of the snake AChR α- subunit and comparing it to that of muscle AChR from other species as well as to neuronal AChR, we may gain insight into the unique pharmacological profile of the snake receptor. The antigenic specificity of the snake muscle AChR was initially tested. Polyclonal rabbit anti Torpedo AChR antibodies cross-reacted with the snake AChR in sections of snake muscle thus indicating that the snake nicotinic AChR shares a certain degree of homology with AChR from other species. To verify that the AChR of the snake was in fact resistant to α-BTX, we examined the effect of intraperitoneal injection of this neurotoxin on snake mortality. Indeed, administration of α-BTX in concentrations several orders of magnitude higher than its LD_{50} for other species did not result in snake death. In search of the snake AChR gene we have prepared Southern blots of snake DNA, digested with several restriction enzymes. By employing a ^{32}P-labeled cDNA probe of the mouse AChR α-subunit (kindly provided by S. Heinemann), we could demonstrate a cross-reaction of this probe with fragments of snake DNA digested with XbaI, BamHI, EcoRI, BglII and HindIII. A genomic library from snake DNA was prepared in a λgt10 vector by isolating genomic DNA fragments in the range of 4-6 Kb, obtained by EcoRI digest of DNA. A positive clone hybridizing to the mouse probe was isolated. The snake AChR thus provides a useful system for elucidating the structural requirements for agonist binding and for discriminating between the α-BTX and the cholinergic binding site in muscle receptor.

ALTERNATE TERMINATION SITES IN HUMAN CHOLINESTERASE GENES

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3. Department of Obstetrics and Gynecology, The Edith Wolfson Medical Center, Holon, The Sackler Faculty of Medicine, Tel Aviv University, Israel.

Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are polymorphic enzymes that exist in parallel arrays of multiple molecular forms with similar kinetic properties. They differ in the number of catalytic subunits, in their level of hydrophobicity and mode of glycosylation, and in their cellular and subcellular localization [1]. We recently isolated a full length cDNA encoding BuChE from fetal human tissues [2,3]. Both the encoded protein and its nucleotide sequence display striking homologies to sequences for Torpedo AChE [4,5]. To examine whether the isolated cDNA also shares homologous sequences with other nervous system cholinesterase (ChE) cDNAs in humans, we searched by screening cDNA libraries for cDNA clones that hybridize with this cDNA but are not identical to it. Libraries were prepared from glioblastoma tissues [6] and neuroblastoma cells [7]. These were found to contain different patterns of ChE molecular forms from those of normal brain tissues [6].

The probes used were the original BuChEcDNA, and oligodeoxynucleotides synthesized according to nucleotide sequences that are homologous in both BuChEcDNA and in AChEcDNA clones from Torpedo californica [4] and Torpedo marmorata [8]. 10^6 phages from each library were screened by both methods. 175 and 200 BuChEcDNA-positive phages were found in the glioblastoma and neuroblastoma libraries, respectively. When screened with the oligodeoxynucleotide probes, similar numbers of positive phages were detected in both libraries, and most of these overlapped with the BuChEcDNA positive phages.

Two glioblastoma clones, however, appeared by comparative hybridization to include the pursued oligonucleotides but to contain different cDNA sequences and were further characterized by DNA sequencing. We found that these were derived from an mRNA transcript that includes ca. 230 nucleotides from the 5' region of the BuChEcDNA, but that it extended beyond the BuChEcDNA at its 5' end and continued with a different sequence in the 3' direction.

About 25% of the hybridization signals obtained with BuChEcDNA were significant but weak, suggesting sequence differences. Out of these, 5 glioblastoma and 3 neuroblastoma clones displayed different restriction patterns from that of the original cDNA. One of these glioblastoma clones and all three neuroblastoma ones contained an additinal 0.5 fragment (designated FI) at the 3' untranslated region of the cDNA. Another one of the glioblastoma clones contained yet another 0.5 kb fragment (designated PII), also at the 3' end. This fragment was not detected in any of the neuroblastoma clones. To establish that the cDNAs containing these fragments were authentic transcripts of the cholinesterase (ChE) genes, and to define their chromosomal location, we employed in situ hybridization to lymphocyte chromosomes [9]. We found that both FI and FII hybridize to the long arm of chromosome 3, similar to the original BuChEcDNA, and that FI also hybridizes to the same area of chromosome 16 to which the original clone hybridizes [10]. Furthermore, FI was also found on a ChE genomic clone from chromosome 3 [11].

The screening results indicate that sequences hybridizing to BuChEcDNA occur in glioblastoma and neurobalstoma cells at a frequency of ca 10^{-4}, which is higher than found in fetal brain, and may be due to the differentiation state of the tissues. We also found that there are multiple termiantion sites in the BuChE gene, and obtained evidence for alternate splicing. Because these phenomena were not detected in cDNA libraries from normal tissues, it will be interesting to see if these changes are related to the different patterns of ChE molecular forms in the glioblastoma and neuroblastoma tissues.

References

1. Massoulie, J. and Bon, S. (1982) Ann. Rev. Neurosci. 5, 57-106.
2. Prody, C. A., Zevin-Sonkin, D., Gnatt, A., Koch, R., Zisling, R., Goldberg, O. and Soreq, H. (1936) J. Neurcsci. Res. 16, 25-36.
3. Prody, C. A., Zevin-Sonkin, D., Gnatt, A., Goldberg, O. and Soreq, H. (1987) Proc. Natl. Acad. Sci. 84, 3555-59.
4. Schumacher, M., Camp, S., Maulet, Y., Newton, M., McPhee-Quigley, K., Taylor, S. S., Friedman, T. and Taylor, P. (1986) Nature, 319, 407-409.
5. Lockridge, O., Bartels, C. F., Vaughan, T. A., Wong, C. K., Norton, S. E. and Johnson, L. L. (1987) J. Biol. Chem. 262, 549-557.
6. Razon, N., Soreq, H., Roth, E., Bartal, A. and Silverman, I. (1984) Exp. Neurol. 84, 681-695.
7. Tumilowicz et al. (1970) Cancer Res. 30, 2110.
8. Sikorav, J. L., Krejci, E. and Massoulie, J. (1987) EMBO J., in press.
9. Soreq, H., Zamir, R., Zevin-Sonkin, D. and Zakut, H. (1987) Human Gen., in press.
10. Zamir, R., Sindel, L., Zakut, H. and Soreq, H., this conference.
11. Gnatt, A. and Soreq, H., this conference.

THE COEXISTENCE AND CORELEASE OF ACETYLCHOLINE AND ENKEPHALINS FROM TORPEDO NERVE TERMINALS

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In the present study we investigated the coexistence of acetylcholine (ACh) and enkephalins in Torpedo electromotor neurons and their corelease following presynaptic stimulation.

Analysis by HPLC of TCA extracts of the electric lobe, electromotor nerve and electric organ of the Torpedo revealed that they contain Met-enkephalin and Leu-enkephalin-like immunoreactivities. The specific concentrations of these opioid peptides is about thirty-fold higher in the electric lobe than in the electric organ (respectively 350 and 12 fmol/mg prot). Subcellular fractionation of electric organ homogenates revealed that ACh and the enkephalins comigrate following differential and density gradient centrifugation and that the purified synaptosomes and the microsomal fraction are highly enriched in both ACh and enkephalins. This suggests that ACh and the enkephalins coexist presynaptically and that the enkephalins are compartmentalized in small vesicles.

K^+ depolarization of isolated electric organ prisms in the prese ce of Ca^{2+} and the acetylcholinesterase inhibitor phospholine iodide results in the corelease of ACh and enkephalin and in the concomitant decrease of the tissue enkephalins content. ACh release proceeds linearly for at least 10 min whereas release of the enkephalins is transient. The extent to which this difference is due to the release process or to hydrolysis of the released enkephalins by endogenous peptidase and enkephalinase activities is discussed.

PHOSPHORYLATION OF THE ACETYLCHOLINE RECEPTOR: STUDIES WITH SYNTHETIC PETIDES AND THEIR ANTIBODIES

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The acetylcholine receptor (AChR) is a 250 kDa transmembrane glycoprotein which consists of four subunits assembled in a molar stoichiometry of $\alpha_2\beta\gamma\delta$. Synthetic peptides corresponding to defined sequences of AChR and their respective antibodies are being employed for the study of receptor phosphorylation. We have previously demonstrated that the cAMP dependent protein kirase (PKA) phosphorylation site resides within sequence 354-367 of the δ subunit of affinity purified as well as membrane bound Torpedo AChR. The phosphorylation site of the Ca^{+2}/phospholipid dependent protein kinase (PKC) was recently shown to be included within the same sequence of the δ subunit. Thus, the phosphorylation sites for both PKA and PKC reside in close proximity, within three consecutive serine residues at positions 360, 361 and 362 of the δ subunit. Phosphorylation by these two kinases may therefore affect biological functioning similarly. Alternatively, these two kinases may operate in an additive, synergistic or antagonistic manner, opening the possibility for pleotropic effects achieved through phosphorylation of the receptor. The role of AChR phosphorylation in receptor function is currently being studied using muscle cells in culture.

RECONSTITUTION OF ISOLATED ACETYLCHOLIN RECEPTORS
- A CRITIQUE -

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"Finkelstein's socks is a decisive parameter for reconstitution of channel proteins into planar bilayers". This sentence nicely caricatures the kind of arguments, used in acetylcholin receptor-channel (AcChR) reconstitution, too. The reason: although some papers suggest the contrary, there are many features of AcChR reconstitution uncontrolled.

AcChR-"molecules" can be purified by affinity chromatography, "reconstituted" into protein-lipid-vesicles and afterwards into planar membranes via, in principle, two methods: fusion or assembly from two vesicle-spread monolayers. In the presence of agonists different kinds of channel openings can be recorded: fast single channels, bursts, and long openings with short closures in between. Similar results have been shown with reconstituted membrane fragments. In addition we see "fuzzy channels" and less defined events of conductivity, which reemerge all the time again. Often , with the same sample, nothing can be seen. Such data have not been published or mentioned yet, because they don't fit into the common idea of how channels have to look like or are thought to be garbage as 99% of the recorded data, too.

Although few articles give evidence, that reconstituted AcChRs can be activated by agonists and blocked by antagonists, the data don't yield more then just evidence, as they lack a profund statistical basis. Besides, single channel recordings are, by principle, not suitable to answer questions like agonist activation.

Reconstitution is a sonorous word for a simple mixing of some molecules. Many people don't realize that, e.g., during dialysis phase separation or structural changes of protein lipid complexes can happen. These partition processes will influence the structure and the composition of the resulting vesicles. Such metastable structures depend on composition and the velocity and sequence of the steps. This phenomena should receive more attention in future.

A major lack of the channel data shown so far is that the number of channels in the measurement cannot be correlated to the number of AcChRs incorporated into the bilayer.

AcChR-channel openings are usually recorded long after application of agonist. Therefore the channels seen in the bilayer cannot be compared with those induced in the synapse; they rather seem to arise from desensitized receptor states. In contrast to the very short distance in the synaptic junction (20 nm) the agonist has to diffuse across a relatively thick unstirred layer (0.1mm) towards the planar bilayer. Therefore the agonist concentration at the membrane will rise from a low concentration in the minute time range, causing desensitisation before an agonist concentration is reached that is high enough for AcChR-channel activation.

To overcome these problems an experimental strategy is proposed which hopefully will evoke a fruitful discussion.

MOLECULAR FORMS AND LOCALIZATION OF ACETYLCHOLINESTERASE AND NONSPECIFIC CHOLINESTERASE IN REGENERATING SKELETAL MUSCLE

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The soleus and EDL muscles of rats were isolated, incubated with an irreversible AChE inhibitor and a myotoxic substance, and autotransplanted back in the place of the contra lateral EDL muscle. Ischemic necrosis was followed by muscle regeneration within the tubes of old basal laminas. Reinnervation of muscle regenerates was prevented by high axotomy of the sciatic nerve.

Molecular forms and histochemical localization of acetylcholinesterase and nonspecific cholinesterase were analysed in muscle regenerates. Regenerating myotubes and myofibres produce the 16S AChE form in the absence of innervation. The asymmetric molecular forms are present throughout the length of muscle regenerates. The 4S globular form predominates in early regenerates whereas the tetrameric 10S AChE form becomes predominant after cross-striatious appear in muscle regenerates. Although the patterns of AChE molecular forms in normal EDL and soleus muscles differ significantly no such differences were observed in noninnervated regenerates from both muscles.

Two types of local accumulation of AChE appear on the sarcolemma of regenerating muscles: first, in places of former motor endplates and, second, in extrajunctional regions. The 'junctional' AChE accumulations appeared also in regenerates prepared from muscles that had been predenervated for one month. AChE in the 'extrajunctional' accumulations can be detached by collagenase treatment of nonhomogenized regenerates indicating the presence of the asymmetric molecular forms. The 4S form of nonspecific cholinesterase is prevailing in regenerating myotubes whereas its asymmetric forms nor focal accumulations could not be identified reliably.

The satellite cells which survive after muscle degeneration probably originate from some type of late myoblasts and transmit the information concerning the ability to synthesize the asymmetric AChE forms and to focally accumulate AChE to regenerating muscle cells. Synaptic basal lamina from former motor endplates may locally induce AChE accumulations in regenerating muscles. The inducing substance does not need a continuous presence of the motor nerve and may be produced by the muscle.

CONFORMATIONAL STABILITY OF NON-AGED AND AGED ORGANOPHOSPHORYL
CONJUGATES OF α-CHYMOTRYPSIN.

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Organophosphqrus (OP) este.s inhibit serine hydrolases such as
chymotrypsin (Cht), trypsin and acetylcholinesterase (AChE), by formation
of a stoichiometric covalent conjugate with the active-site serine.

The inhibited enzyme can subsequently undergo one of two competing
processes: either reactivation or 'aging', with the latter resulting in
a conjugate which is completely resistant to reactivation (Hobbiger, 1955;
Aldrige & Reiner, 1972). Aging involves detachment of a substituent from
the phosphorus atom, with concomitant introduction of a negative charge
into the active-site, according to the following scheme:

inhibition

$$DI^{+}XP(O)(OR')R \xrightarrow{\quad -U\text{b}- \quad} EP(O)(OR')R \xrightarrow{\quad -B'- \quad} EP(O)(O^-)R$$

reactivation   aging
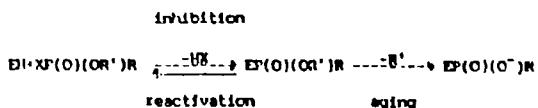
where R is an alkyl, alkoxy or aryloxy group, R' is an alkyl or aryl group,
and X is a leaving group. Kinetic studies show that the electrostatic barrier
provided by the negative charge cannot, alone, explain the complete resistance
to reactivation observed experimentally (Behrman et al., 1970).

The aim of this study was to investigate the susceptibility to confor-
mational changes of the active-site region of non-aged and aged Cht.

The susceptibility to denaturation of pyrenebutylethylphosphoryl-Cht
(FBEP-Cht) (non-aged) and of pyrenebutylhydroxyphosphoryl-Cht (PBP-Cht)
(aged) was studied by measurement of 'heir circular dichroism (CD) spectra
at various concentrations of guanidinium hydrochloride (Gu.HCl). Reactiva-
tion studies, after incubation with Gu.HCl, were performed in parallel.

The CD spectrum of PBEP-Cht exhibits four conformational transitions:
The first, at 0.3-0.4 M Gu.HCl, in the range of 310-340 nm; the second, at
about 0.7 M Gu.HCl, near 350 nm; the third, at 1-2 M Gu.HCl, in the range
of 290-310 nm; and the fourth, at 2-3 M Gu.HCl, in the range of 215-290 nm.
In contrast, PBP-Cht exhibits only two conformational transitions: The
first, at ca. 0.7 M Gu.HCl, is observed at about 350 nm; the second, at
2-3 M Gu.HCl, is observed in the whole spectral range of 215-370 nm.

The optical activity above 310 nm originates in the pyrene chromophore;
it thus reflects the conformation of the catalytic site, whereas the tran-
sition at ca. 235 nm may be ascribed primarily to the polypeptide linkages
of the whole protein. In both conjugates conformational changes in the

pyrene absorption region occur at Gu.HCl concentrations which are much lower
than that required for total denaturation of the enzyme, which is 2-3M, as
was also established in control experiments with unmodified Cht. However,
reactivation studies which were performed with PBEP-Cht at the various Gu.CHl
concentrations demonstrated that its ability to undergo reactivation with
3-pyridinealdoxime methiodide (3 PAM) (Cohon & Erlanger, 1960) is maintained
as long as total denaturation has not been achieved; the catalytic activity
of unmodified Cht is similarly maintained unless total denaturation is achieved.
Thus, there appears to be no disruption of the active sites of either the
OP-Cht conjugates or of the unmodified enzyme prior to full denaturation.
We may, therefore, conclude that all the conformational transitions observed
above 310 nm and at Gu-HCl concentrations below 2M result from different
orientations of the pyrene probe within the active site, rather than from
disruption of the active site itself. It should be noted, however, that there
is a marked difference between the tendency of PBEP-Cht and that of PBP-Cht
to undergo orientational changes within their active sites. The non-aged
conjugate undergoes more such reorientations, the first occuring at a Gu.HCl
concentration as low as 0.3M; in contrast, the first observed orientational
change observed for the aged conjugate, PBP-Cht, occurs only at 0.7M Gu.HCl.
These results clearly support the contention that the aged form has undergone
a conformational stabilization in its active site, relative to the non-aged
form.

REFERENCES

Aldrige, W.N. & Reiner, E. (1972) Enzyme Inhibitors as Substrates, Elsevier,
    Amsterdam.

Behrman, E.J., Biallas, M.J., Brass, H.J., Edwards, J.O. & Isaks, M.
    (1970) J. Org. Chem. 35, 3069.

Hobbiger, H. (1955) Brit. J. Pharmacol. 10, 356.

PHYSICAL PARAMETERS OF THE TRANSFORMATIONS AND DISTRIBUTION OF INFORMATIONS.

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Action Potentials and electronic potentials are conducted to the limits of the cell only, with the exception of some very specialized structures where the adjacent cells are not affected by potential changes in certain cell. The nervous system, however, is a network with strong functional coupling of cells and in addition information has to be transmitted from receptor cells to nerve cells, and from these to effectors like muscle or gland cells. The transmission occurs at synapses, at morphologically highly specialized contacts between cells. In most cases a chemical transmitter substance mediates the information transfer.

The action potential reaching the nerve terminal induce the release of the content of synaptic vesicles into the synaptic cleft. The vesicles contain a transmitter substance which in case of the neuromuscular junction is acetylcholine. This transmitter diffuses across the synaptic cleft and reacts with receptors in the postsynaptic membrane, which cause the opening of synaptic ion channels. The resulting ionic currents generate postsynaptic potentials which may trigger action potentials in the postsynaptic cell, completing the synaptic transmission process.

A number of chemical transmitters are known which act at different synapses. The best investigated ones are: Acetylcholine, Epinephrine/Norepinephrine, γ-amino butyric acid and glutamate. For these substances the release of transmitter after stimulation was proven chemically. For the funtional role of the following transmitters some final evidence is still missing: glycine, asparate, serotonine, dopamine, nucleotides like adenosine and some peptides which also may act as hormones. All these molecules are relatively small and some of them occur in the metabolism of many cells at relatively high concentration. These substances only can act as transmitters at locally very high concentrations and by means of special supportive mechanisms. Very probably many more transmitters will be found in addition to the listed ones.

The mode of release corresponds to an electrophysiological observation which is obtained in all synapses studied thoroughly. Postsynaptic potentials or currents are composed of small units of quanta. To understand the mechanism of release of transmitters, synaptic current will be proposed to measure in different conditions.

The release of transmitter by the presynaptic neuron is induced by the action potential. It is not necessary, however, that the neuron is excited also passive depolarization leads to release of transmitter. At the giant synapse the presynaptic potential can be varied by application of current. The release of transmitter depends very much on the Ca^{2+} concentration in the extracellullar fluid. The postsynaptic potential increases about proportional to the 4th power of Ca^{2+} concentration. This relationship may indicate that the release of one quantum of transmitter is induced by the cooperative reaction of four Ca^{2+} with regulatory sites in the memberane. An increase of the intracellular Ca^{2+} concentration directly before the release of transmitter has been already measured. We have planned to study the effect of other anions on the release of transmitters. We have also proposed to measure the excitatory postsynaptic potentials and inhibitory postsynaptic potentials which depolarize and hyperpolarize the memberane respectively.

MOLECULAR FORMS OF ACETYLCHOLINESTERASE IN

ADULT DROSOPHILA : STRUCTURE AND HYDROPHOBIC INTERACTIONS

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Drosophila acetylcholinesterase (AChE, EC 3117) from crude extracts of adult heads or after purification by affinity chromatography was studied by ultracentrifugation in sucrose gradients and by non denaturing electrophoresis in the presence of Triton X100, Brij 96 or sodium deoxycholate.

The major molecular form of AChE sedimented at 6.1 S in the presence of Triton X100, at 4.2 S in the presence of Brij 96 and formed fast sedimenting aggregates in the absence of detergent. Treatments of this form by proteases or phosphatidylinositol phospholipase C (PIPLC) suppressed the interaction with detergents but the catalytic activity of the lytic form remained unaffected. The native 6.1 S form is therefore referred to as an amphiphilic enzyme.

Autolysis (storage at +4°C for several weeks) converted the amphiphilic form into two components (sedimenting at 6.5 S and 4.2 S) which no longer interacted with detergents. These 6.5 S and 4.2 S forms were always present in minor amount in rapidly-extracted material (in the presence of antiproteolytic agents).

Partial disulfide bridge reduction of the amphiphilic 6.1 S form produced a catalytically active component sedimenting at 3.5 S in Triton X100, at 2.0 S in the presence of Brij 96 and forming aggregates in the absence of detergent.

These results, as well as the data from non denaturing electrophoresis (illustrated on the poster) demonstrate that the major molecular form of Drosophila AChE is a disulfide-linked amphiphilic dimer (G2) which can be converted into a detergent interacting monomer (G1) by reduction or into hydrophilic G2 and G1 on autolysis. The hydrophobic domain of amphiphilic G2 and G1 is constituted by a glycolipid (see also Gnagey et al., J Biol Chem, in press).

The figure summarizes these conclusions:
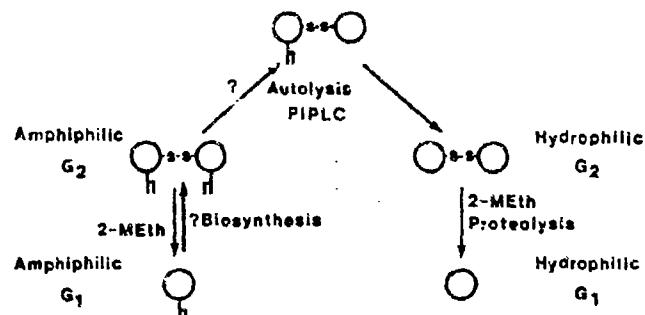
References:

Fournier D, Bergé J, Bordier C. AChE from Musca domestica and Drosophila melanogaster heads are linked to membranes by a glycophospholipid anchor sensitive to an endogenous phospholipase. J Neurochem, in press.

Toutant JP, Arpagaus M. Fournier D. Native molecular forms of head AChE from adult Drosophila melanogaster: Quaternary structure and hydrophobic character. J Neurochem, in press.

MONOCLONAL ANTIBODIES TO CHICKEN ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE

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The asymmetric (20 S) acetylcholinestease (AChE) from one-day old chick muscle was purified on an immunoaffinity column of immobilized antibody to chick brain AChE and used for immunization. Eight monoclonal antibodies (mAbs) against the muscle enzyme were isolated and characterized. Five antibodies (4A8, 1C1, 10B7, 7G8 and 8H11) recognised the 110-kDa AChE catalytic subunit, one antibody (7D11) recognised the 72-kDa butyrylcholinesterase (BuChE) catalytic subunit and 2 antibodies (6B6 and 7D7) reacted with the 58-kDa collagenous tail unit. Those 3 polypeptides can be recognised in the 20 S enzyme used, which is a hybrid enzyme. Four of the anti-110-kDa subunit mAbs stained the AChE on immunoblots. Sucrose density gradient analysis of the antibody enzyme complexes showed that the anti-110-kDa subunit mAb cross-links the 20 S AChE molecules and migrates to the bottom of the tube, while there is only a 2 - 3 S shift with the mAbs specific for the 72-kDa or the 58-kDa subunit, suggesting that these subunits are more inaccessible in the structure for cross-linking by one IgG molecule. The 4A8, 10B7, 7D11 and 7D7 mAbs showed cross-reactivity to the corresponding enzyme from quail muscle; however, none of the eight mAbs reacted with either enzyme from mammalian muscle and Torpedo electric organ. The antibodies showed immunocytochemical localization of AChE at the neuromuscular junction of chicken and quail. Using the AChE- and BuChE-specific mAbs, the amounts of homogenous BuChE and hybrid AChE/BuChE asymmetric enzymes in the 1-day old muscle crude extracts could be compared.

GLYCOLIPID ANCHORAGE OF A TAILED ASYMMETRIC 16S ACETYLCHOLINESTERASE VARIANT IN RAT SUPERIOR CERVICAL GANGLION NEURONAL MEMBRANES.

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Tailed asymmetric 16S acetylcholinesterase (AChE ; EC 3.1.1.7) is present in rat superior cervical ganglion (SCG) neurons in vivo and in vitro. Two pools of 16S AChE can be revealed by differential extraction : one, classically, by high salt (HS) and another one by detergent treatment. The detergent extractible (DE) 16S AChE constitutes a significant proportion of total SCG neuronal 16S AChE (30 %). Upon preincubation in the presence of Bacillus Cereus phospholipase C (EC ; 3.1.4.3.) DE 16S AChE is quantitatively released from a SCG membrane preparation suggesting that it exists a specific pool of 16S AChE attached to the neuronal membranes through a phospholipid anchor.

Moreover, exposure of the membranes to a phosphatidyl inositol specific phospholipase C (PiPLC ; monophosphatidyl inositol phosphodiesterase ; EC 3.1.4.10) results in the release of up to one-third of the DE pool of 16S AChE. Thus, at least part of DE 16S AChE is anchored to neuronal membranes through a phosphatidyl-inositol glycolipid. Furthermore, by studying 1) SCG pretreated by an irreversible AChE inhibitor, methylphosphorothiolate (MPT) in the presence of a reversible non-permeant inhibitor (BW 284 C 51 compound) in order to protect extracellular AChE and 2) primary neuronal SCG cultures in which 16S AChE is essentially internal, we found that both external and internal AChE had a pool of DE 16S AChE released by PIPLC. Thus, a phosphatidyl inositol glycolipid can anchor both external and internal DE 16S AChE.

These results suggest an internal assembly of PI containing residues with the AChE polypeptides and makes plausible the occurrence of differential splicing of mRNAs originating from a single AChE gene.

CHOLINESTERASE SYNTHESIS IN DEVELOPING HUMAN OOCYTES REVEALED BY IN SITU HYBRIDIZATION TO FROZEN OVARIAN SECTIONS

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In addition to their well known involvement in neuromuscular junctions and in brain cholinergic synapses, cholinergic mechanisms have been implicated in the growth and maturation of oocytes and in fertilization processes in various species [1]. Functional acetylcholine receptors were electrophysiologically demonstrated in amphibian and mammalian oocyte membranes [2,3] and cholinergic antagonists were shown to interfere with fertilization [4]. The neurotransmitter acetylcholine was detected in mammalian sperm cells [5] and activity of the acetylcholine hydrolyzing enzyme, acetylcholinesterase (AChE) was biochemically measured in the exceptionally big oocytes of the frog Xenopus laevis [6,7]. However, biochemical methods could not reveal whether AChE was produced within the oocytes themselves or in the surrounding follicle cells. Furthermore, this issue is particularly important for understanding growth and fertilization processes in the much smaller human oocytes, in which the sensitivity of AChE biochemical measurements is far too low to be employed.

To directly determine whether the human cholinesterase (ChE) genes are transcriptionally active in oocytes, and, if so, at what stages in their development, the presence of ChEmRNA was pursued. For this purpose frozen ovarian sections were subjected to in situ hybridization using (35S)-labeled human ChEcDNA. Highly pronounced hybridization signals were localized within oocytes in primordial, pre-antral and antral follicles, but not in other ovarian cell types, demonstrating that within the human ovary ChEmRNA is selectively synthesized in viable oocytes at different developmental stages. Sucrose gradient centrifugation followed by (3H)-acetylcholine hydrolysis measurements revealed in the ovarian extracts the presence of low levels of soluble AChE dimers, sensitive to the specific AChE inhibitor BW284C51 but resistant to the BuChE inhibitor iso-OMPA [8]. In view of the low numbers of oocytes out of total cells in the ovary, these findings strongly suggest that AChE is a prominent protein in human oocytes throughout their development and further support the hypothesis that cholinergic mechanisms may be involved in oocyte growth and maturation and/or in sperm-egg interaction in human.

Furthermore, the pronounced synthesis of ChE transcripts in oocytes suggests that the ChE genes are particularly good candidates for the formation and re-insertion of inheritable processed genes.

References

1. Eppig, J. J. and Downs, S. M. (1984) Biol. Repr. 30, 1-11.
2. Kusano, K., Miledi, R. and Stinnakre, J. (1977) Nature 270, 739-741.
3. Eusebi, F., Mangia, F. and Alai, L. (1979) Nature 277, 651-653.
4. Florman, H. M. and Storey, B. T. (1981) Exp. Zool. 216, 159-167.
5. Sastry, B. V. R. and Sadavongvivad, C. (1979) Pharmacol. Rev. 30, 65-132.
6. Soreq, H. (1985) CRC Crit. Rev. Biochem. 18, 199-238.
7. Gundersen, C. B. and Miledi, R. (1985) Neurosci. 10, 1487-1495.
8. Malinger, G., Zakut, H. and Soreq, H. (1987), submitted.

CHROMOSOMAL MAPPING OF HUMAN CHOLINESTERASE GENES BY IN SITU HYBRIDIZATION

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In order to map the structural human ChE genes to distinct chromosomal regions,
a cloned human cDNA for cholinesterase (ChE) [1] was used a a probe for in situ
hybridization to chromosomes. For this purpose, lymphocyte chromosome spreads were
prepared following short-term culture, and hybridized with ChEcDNA fragments labeled with
[^{35}S] by different techniques. These included direct labeling of electrophoretically
purified cDNA inserts by nick-translation, synthesis of a labeled second DNA strand to
this cDNA subcloned into the single-stranded M13 phages, in vitro transcription of such
cDNA into labeled RNA and the use of random oligonucleotides as primers for DNA polymerase
I synthesis. Following the hybridization procedure [2], chromosome spreads were exposed
under photography emulsion to create silver grains in sites of hybridization and were then
R-banded to enable karyotype analysis and to localize specific silver grains on particular
chromosome regions. Using this analysis, the recent genetic linkage assignment of the ChE1
gene to the long arm of chromosome 3 [3] was confirmed and further refined to 3q21-q26, in
close proximity to the genes coding for transferrin (TF) [4] and transferrin receptor
(TRFC) [5]. The CHE1 locus localizes to a 3q region that is commonly abberrated, and
related with abnormal megakaryocyte proliferation, in acute myelodysplastic anomalies [6].
In view of earlier findings that ChE inhibitors induce megakaryocytopoiesis in culture
[7,8], this localization may indicate that ChEs are involved in regulating the
differentiation of megakaryocytes. Cummulative histograms of in situ hybridization of
ChEcDNA to chromosome No. 3 further suggest that there might be two sites carrying ChE
sequences on the long arm of this chromosome.

A second site for ChEcDNA hybridization was found on chromosome 16q11-q23,
demonstrating that the CHE2 locus of the cholinesterase gene, which directs the production
of the common C5 variant of serum ChE [9], also codes for a structural subunit of the
enzyme and is localized on the same chromosome with the haptoglobin (HP) gene [10], both
genes found on the long arm of chromosome 16. Further in situ hybridization experiments
using human haptoglobin cDNA (gratefully received from Drs. S. Oliviero and G. Cortese)
revealed that these two genes are at least 1000 kb apart from each other. The finding of
various sites for ChEcDNA hybridization [12] suggests that the different loci coding for
human ChEs may include non-identical sequences, responsible for the biochemical
differences between ChE variants.

References

1. Prody, C., Zevin-Sonkin, D., Gnatt, A., Goldberg, O. and Soreq, H. (1987) PNAS 84,
   3555-3559.
2. Rabin, M., Fries, R., Singer, D. and Ruddle, F. H. (1985) Cytogenet. Cell Genet. 39,
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3. Arias, S., Rolo, M. and Gonzalez, M. (1985) Cytogenet. Cell Genet. 40, 571.
4. Huerre, C., Uzan, G., Greschik, K. H., Weil, D., Levin, M., Hors-Cayla, M. C., Boue,
   J., Kahn, A. and Junien, C. (1984) Ann. Genet. 27, 5-10.
5. Kidd, K. K. and Gusella, R. (1985) Cytogenet. Cell Genet. 40,107-127.
6. Bernstein,R., Pinto, M. R., Behr, A. and Mendelow, B. (1982) Blood 60, 613-617.
7. Burstein, S. A., Adamson, J. W. and Waker, L. A. (1980) J. Cell. Physiol. 103,
   201-208.
8. Paulus, J. P., Maigen, J. and Keyhani, E. (1981) Blood 58, 1100-1106.
9. Lovrien, E. W., Magenis. R. E., Rivas, M. L., Lamvik, N., Rowe. S., Wood, J. and
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10. Simmers, R. N., Stupons, I. and Sutherland, G. R. (1986) Cytogenet. Cell Genet. 41,
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11. Mattei, M. G., Phillip, N., Passage, E., Moisan, J. P., Mandel, J. L. and Mattei, J.
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ANTIBODIES TO CLONE-PRODUCED HUMAN CHOLINESTERASE: INTERACTION WITH DENATURED AND NATIVE CHOLINESTERASES AND WITH THYROGLOBULIN

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In order to study the polymorphism of human cholinesterases (ChEs) at the levels of primary sequence and structure, a fragment of human butyrylcholinesterase (BuChE)cDNA [1] was subcloned into the pEX bacterial expression vector [2] in conjunction with the β-galactosidase gene. The production of fusion protein from this plasmid was induced and its polypeptide product analyzed. Gradient gel electrophoresis followed by immunoblot analysis revealed that the clone-produced BuChE peptides interact specifically with antibodies against human and Torpedo acetylcholinesterase (AChE) (gratefully received from Drs. U. Brodbeck and P. Taylor, respectively). Rabbit polyclonal antibodies were then prepared against the purified clone-produced BuChE polypeptides and were subjected to immunoblots with denatured serum BuChE as well as with purified and denatured erythrocyte AChE (gratefully received from E. Schmell). In contrast, these antibodies interacted differently with various ChEs at their native forms. Native BuChE tetramers from human serum, but not AChE dimers from erythrocytes, interacted with these antibodies to produce antibody-enzyme complexes which could be precipitated by second antibodies and which sedimented faster than the native enzyme in sucrose gradient centrifugation. Similar analysis revealed changes in the sedimentation properties of both AChE and BuChE dimers from muscle extracts, but not of any other form of muscle ChE. These findings demonstrate that the polymorphic human ChEs share sequence homologies and suggest the existence of considerable structural differences between various molecular forms of ChE within particular tissues, as well as between similarly sedimenting molecular forms of ChE from different tissues.

In addition to the homologies between various ChEs from different species, molecular cloning studies have recently revealed impressive sequence homologies between bovine thyroglobulin (Tg) [3] and Torpedo [4] and human [5] ChEs, suggesting that hyperthyroidism-induced autoantibodies to Tg in the serum of Grave's ophthalmopathy patients may cross-interact with ChEs and induce cholinergic symptoms [6]. To examine this hypothesis, the polypeptide products of human ChEcDNA expressed in bacteria were separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose and incubated with rabbit anti-Tg antibodies, as well as with IgG fractions from the serum of 9 patients suffering from Grave's ophthalmopathy and 6 clinically healthy controls. Incubation with ^{125}I-protein A and autoradiography revealed specific immunoreaction between the rabbit anti-Tg antibodies and human ChE-derived peptides. Antibodies to the ChE peptides were clearly detected in the serum of 7 out of the 9 patients but not in the controls. Reciprocal analyses with the rabbit antibodies elicited against the human ChE-derived peptides and against purified rat brain AChE [7] (gratefully received from J. Massoulie and M. Vigny), revealed that both antibodies interact specifically with purified human Tg (in collaboration with M. Ludgate and G. Vassart).

To further reveal whether the anti-ChE antibodies may interact in situ with ChEs in neuromuscular junctions, bundles of muscle fibers were microscopically dissected from the region in fetal human diaphragm which is innervated by the phrenic nerve. Muscle fibers incubated with the examined antibodies and with ^{125}I-protein A were subjected to emulsion autoradiography, followed by cytochemical ChE staining. The anti-ChE antibodies created patches of silver grains in the muscle endplate region stained for ChE. To this end, the anti-cloned ChE antibodies, as well as six of the above described patient sera and anti-Tg antibodies, were examined and found positive, using this technique. This was performed under conditions where control sera do not induce the appearance of silver grains at endplate regionss. Our findings demonstrate that the sequence homology between Tg and ChE is physiologically significant, and suggest that autoantibodies to Tg may cross react with ChEs and be implicated in creating cholinergic symptoms in Grave's ophthalmopathy patients.

References

1. Prody, C., Zevin-Sonkin, D., Gnatt, A., Koch, R., Zisling, R., Goldberg, O. and Soreq, H. (1986) J. Neurosci. Res. 16, 25-36.
2. Stanley, K. K. and Luzio, J. P. (1984) The EMBO J., 3, 1429-1434.
3. Mercken, L., Simons, M. J., Swillens, M., Massaer, M. and Vassart, G. (1985) Nature, 316, 647-651.
4. Schumacher, M., Camp, S., Maulet, Y., Newton, M., McPhee-Quigley, K., Taylor, S. S., Friedman, T. and Taylor, P. (1986) Nature 319, 407-409.
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6. Ludgate, M., Swillens, S., Mercken, L. and Vassart. G. (1986) Lancet II, 219. 1986.
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